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NANOfeatures- Focus nas características de NANOpartículas: modelação da exposição e toxicidade

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Mónica João de Barros Amorim, Investigadora Principal do Departamento de Biologia da Universidade de Aveiro e Doutor Janeck James Scott-Fordsmand, Investigador Sénior do Departamento de Biociências da Universidade de Aarhus, Dinamarca.

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Em memória de António Manuel da Gama Mendes.
Por todas as questões colocadas e por todas as que ficaram por responder.

o júri

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palavras-chave

nanomateriais; tempo de exposição; cobre; prata; solo; colêmbolos; revestimento; interação entre espécies; diferentes níveis organizacionais; iões; efeitos transgeracionais; reprodução; tamanho; stress oxidativo; predação; abundância; acumulação.

resumo

Nanomateriais (NMs) tomaram um importante papel no dia-a-dia, devido às várias aplicações em diferentes áreas (biomedicina, engenharia, entretenimento…). Ainda que úteis para o Homem, o potencial perigo dos muitos NMs produzidos ainda não foi completamente avaliado.

A maioria dos resíduos antropogénicos acabam no solo através de uma variedade de rotas, ficando disponível para as espécies que compõem os ecossistemas de solo. A potencial toxicidade destes NMs para os ecossistemas de invertebrados de solo representa uma lacuna de conhecimento no design e uso de NMs mais seguros que deve ser colmatada.

Nesta tese, o organismo modelo *Folsomia candida* foi exposto a diferentes NMs metálicos, pristino e modificados, e aos respectivos sais, em meios cada vez mais complexos, desde testes estandardizados a sistemas multi-espécies de solo (SMS), e períodos de exposição ampliados, reflectindo cenários reais. Foi usada uma abordagem multi-paramétrica em diferentes níveis organizacionais (celular, organismo, população até ao nível do ecossistema), avaliando as contribuições de quatro principais conceitos para o desenvolvimento de toxicidade de NM: tempo, transformação no meio de exposição, modificação dos NMs e interacções entre espécies.

Devido à exposição a AgNM e $AgNO_3$ durante intervalos de tempo de 10 dias e estandardizados (28 dias), foram activadas defesas antioxidantes e aumentou a produção de metalotioneína, com uma resposta mais tardia à forma nano que a não-nano, enquanto não foram observadas diferenças significativas na reprodução após exposição a NM. Numa exposição contínua multigeracional (6 gerações – 186 dias) a $AgNO_3$, foi demonstrado efeito na reprodução, mesmo após transferência para solo limpo. Com o aumento do número de gerações, foram observados efeitos transgeracionais, com uma diminuição no tamanho dos organismos, inferindo uma adaptação em condições adversas para evitar a extinção.

Em testes SMS, verificou-se que os efeitos tóxicos de CuO NMs na abundância das espécies aumentaram em exposições a longo prazo (84 dias), enquanto que a forma sal de cobre ($CuCl_2$) resultou num impacto mais imediato. A modificação de CuO NMs com diferentes revestimentos resultou em diferentes níveis de resposta entre as espécies e dinâmicas do ecossistema a longo prazo, correlacionado com o potential zeta adquirido. Em geral, os NMs revestidos e com carga negativa foram mais tóxicos que que os NMs revestidos e com carga positiva, enquanto que NMs sem carga produziram efeitos num intervalo de tempo mais curto. Foi demostrado que as interacções entre espécies e exposição a NMs têm efeitos sinergísticos na composição e dinâmicas do ecossistema. Enquanto que a quase totalidade de cobre se encontrou ligada à matriz do solo, a concentração interna de cobre em *F. candida* variou, tanto na concentração máxima como com o tempo, de acordo com o revestimento do NM.

Em conclusão, a toxicidade de NMs metálicos aumenta com o tempo e, enquanto alterações celulares são verificadas em exposições curtas, o seu impacto a níveis organizacionais superiores é apenas visível em exposições longas. Sugere-se o aumento período de tempo dos testes estandardizados para incluir a avaliação de toxicidade de NMs.

keywords

nanomaterials; exposure time; copper; silver; soil; collembola; coating; species interactions; different organizational levels; ions; transgenerational effects; reproduction; size; oxidative stress; predation; abundance; accumulation.

abstract

Nanomaterials (NMs) have taken an important role in everyday life, due to several applications in different areas (biomedical, engineering, entertainment…). While useful to man, the hazardous potential of the many NMs produced has not been fully assessed.

Most of the anthropogenic waste end up in the soil by a variety of routes, becoming available to the species that make up the soil ecosystems. The potential toxicity of these NMs to soil invertebrate ecosystems represents a knowledge gap in the design and use of safer NMs that must be challenged.

In the present thesis, the standard model organism *Folsomia candida* was exposed to different metal-based NMs, either pristine or modified, and its salt counterparts in increasing complex media, from standardized testing to soil multispecies systems (SMS), and increasingly exposure period, reflecting real-life scenarios. A multi-endpoint approach on several organizational levels (cellular, organism, population up to a full ecosystem level) was used, assessing the contributions of four main concepts for the development of NM toxicity: time, transformation in exposure media, modification of the NMs and species interactions.

Due to exposure to AgNM and $AgNO_3$ in a 10 days and a standardized (28 days) timeframe, antioxidant defences were activated and metallothionein production increased, with a later response to nano than to non-nano form, while no significant differences in reproduction were observed after NM exposure. In a multigenerational continuous exposure (6 generations - 186 days) to $AgNO_3$, an effect on reproductive output was shown, even after transfer to clean soil. With the increasing number of generations, transgenerational effects were observed, with a decrease in the size of the organisms, inferring an adaption in adverse conditions to avoid extinction.

In the SMS testing, it was observed that CuO NMs toxic effects on species abundance increased on a long-term exposure (84 days) while copper salt form ($CuCl_2$) resulted in a more immediate impact. The modification of CuO NMs with different coatings resulted in different response degrees between species and the ecosystem dynamics on the long term, correlated with the acquired zeta potential. Overall, the negative charged NMs were more toxic than positive charged NMs, while non-charged NMs produced effects on a shorter timeframe. It was shown that species interactions and the NM exposure have a synergistic effect on the ecosystem composition and dynamics. While virtually all of the copper was attached to the soil matrix, *F. candida* internal copper concentration varied both in maximum concentration and with time, according the NM coating.

Concluding, metal-based NMs toxicity increases with time and while cellular changes are shown in short exposures, their impact on higher organizational levels are only shown on longer exposure times. An increase in the standardized testing timeframe is suggested to accommodate NM toxicity assessment.

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List of Abreviations:

AAS-GF – Graphite Furnace Atomic Absorption Spectroscopy

Ag - Silver

AgNM300K – Silver Nanomaterial

AgNP – Silver Nanoparticle

$AgNO_3$ – Silver Nitrate

ANOVA – Analysis of Variance

ASC – Ascorbate

CAT – Catalase

Cd - Cadmium

CEC - Cation Exchange Capacity

CIT – Citrate

Cu - Copper

$CuCl_2$ – Copper Chloride

CuO – Copper Oxide

CuO NM – Copper Oxide Nanomaterial

CV – Coefficient of Variation

DW – Dry weight

EC_{50} – Half Maximal Effective Concentration

F_n – Juvenile Generation N

GPx – Glutathione Peroxidase

GR – Glutathione Reductase

GSH – Reduced Glutathione

GST – Glutathione-S-Transferase

HC - Hazard Concentration

IS – Individual Species

ISO – International Organization for Standardization

LPO – Lipid Peroxidation

MG - Multigenerational

Na^+ – Sodium Ion Form

NM – Nanomaterial

NP - Nanoparticle

OECD – Organisation for Economic Cooperation and Development

P_n – Parental Generation N

PEI - Polyethylenimine

PRC - Principal response curve

PVP - Polyvinylpyrrolidone

RDA – Redundancy Analysis

ROS – Reactive Oxygen Species

SMS – Soil Multispecies System

SSD - Species Sensitive Distribution

TG – Total Glutathione

WHC - Water Holding Capacity

ZnO – Zinc Oxide

Chapter One

Introduction

Nanomaterials and Nanotechnology

Nano is a prefix that, by definition, means one billionth of the unit ($1x10^{-9}$). Since Richard Feynman described the field (Feynman, 1960) computers have reduced in size from being as big as a room to being fitted to our wrist. In the 1970s and 1980s the first focus on Feynman's ideas was developed, with Taniguchi in 1974 and Drexler in 1986 coining the terms nano-technology (Taniguchi, 1974) and nanotechnology (Drexler, 1986). Today, nanotechnology is the development and production of material at the nanoscale

The definition of nanomaterials (NMs), as recommended by the European Commission in 2011 (European Comission, 2011) and currently under revision based on the Joint Research Council recommendations from 2015 (European Commission et al., 2015) is as follows:

"A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.

In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %."

Since the early 2000s there has been an exponential growth of the global market in the area of nanotechnology, having rose from a volume of €200 billion in 2009 to €2 trillion in 2015 (European Comission, 2018a). Engineered nanomaterials (ENMs) present tremendous opportunities for industrial growth and development, holding great promise for the enrichment of the lives of citizens (Forster et al., 2011; European Comission, 2018b).

The larger number of nanomaterial-based products are related to cosmetics and textiles (Foss Hansen et al., 2016). Also, many medical and technical applications such as tumour therapies, batteries for electrical cars and solar panels are being developed. These developments have the potential to create major technological breakthroughs. As such, nanomaterials have been identified as a key enabling technology (Stark et al., 2015; European Comission, 2018a).

However, in sight of this huge development and the resulting number and variety of new nanobased-materials, there is a large gap in the potential nanomaterial toxicity and impact in environment and public health (Hu et al., 2016). Along with the need for methods that ensure the engineering of safe-by-design nanomaterials, the development of models to predict the impact of nanomaterials have been the biggest challenges in the field for the past decade and a half (Maynard et al., 2006).

Applications and Characterization of Tested Nanomaterials

The physical and chemical characterization of nanomaterials is an essential aspect for any study regarding environmental or health impact. Which characteristics are of importance to classify these materials and later test has been focus of discussion in recent years (Stone et al., 2010).

Main Applications of Nanomaterials

Silver (Ag)-based NMs are of the most used worldwide, with an estimated production of 55 tons/year, as of 2012 (Piccinno et al., 2012). Textiles make up 30-50% of their usage, with other purposes being identified such as cosmetics, cleaning agents, consumer electronics and medical devices. Their anti-microbial properties result in their high usage, as well as their conductivity properties.

In regard to copper (Cu)-based NMs, electrical applications such as printing devices and acting as biocide for wood preservation are the main usages (Pang et al., 2012; DaNa2.0, 2018). In 2008, 50% of the North American wood protection market used Cu-based NMs for its purpose, in a resulting 39500 tons/year (Evans et al., 2008).

Nanomaterial Characterization

While size is the one characteristic that defines the “nano” in nanomaterial, there are several other physical and chemical properties that help characterize the tested NMs and that have an influence on their interaction in exposure and test media. Another relevant property that contributes to the interest in NM versus bulk form is the increased surface area to volume ratio. This will increase the number of possible surface interactions and therefore the activity of the NM (Oberdörster et al., 2005) and its effect on organisms.

Several other properties such as surface charge, shape, crystal structure, coating/corona, aggregation / agglomeration and solubility / dissolution, amongst others have been shown to be relevant to NM activity (Gatoo et al., 2014).

This is of importance when assessing the hazard / risk level of the NMs as the number of synthesis protocols and nanomaterial variety has greatly increased in the last decade and a half. The need for proper NM characterization, not only in their pristine form but also when modified or in exposure, is a consensual position today (Hristozov et al., 2016; Nowack, 2017; Scott-Fordsmand et al., 2017).

Although there has been an increase in the number of methods that allow NM characterization (for more details see Table 1), there is still a lack of adequate quantitative methods. Currently the analysis for several parameters is done by coupling different techniques according to the media and aim of the study, while some techniques like single particle ICP-MS (SP-ICP-MS) have become essential in the nanoscale analysis.

Furthermore, in light of novel techniques, in recent years there has been long discussions on the standardization of characterization methods and parameters (Tämm et al., 2016), looking towards the need to fulfil regulatory compliance.

However, these regulatory and by-law limitations should not be mistaken with the level that scientists aim for.

Table 1: List of physical properties/metrics and chemical composition/analytes of NMs and respective associated instruments and methods (Hassellöv et al., 2008; Contado, 2015; Laborda et al., 2016)

Physical properties/metrics ***Instruments and methods***	
Diameter	TEM, FESEM, ESEM, AFM, FFF, DLS, NTA, VIP, PCC
Volume	Sd-FFF
Area	TEM, ESEM, AFM
Mass	LC-ESMS, VIP, ICP-MS, SP-ICP-MS, AF4
Surface charge	Zeta-Potential, Electrophoretic Mobility
Crystal structure, Aspect ratio or other shape factor	TEM, STEM, FESEM, ESEM, XRD, TEM-XRD-SAED, SP-ICP-MS,
Chemical composition/analytes	
Elemental composition	Bulk: ICP-MS, ICP-OES, AAS-GF, XAS, VIP Single nanoparticle: TEM-EDX, SP-ICP-MS Particle population: FFF-ICP-MS, VIP
Fluorophores	Fluorescence Spectroscopy
Fullerene (‘‘molecules’’)	UV-vis, IR, NMR, MS, HPLC
Total organic carbon	High Temperature Chemical Oxidation
Other properties not falling within the above classes	
Aggregation state	DLS, AFM, SP-ICP-MS, ESEM
Hydrophobicity	LLEC
Dissolution rate	VIP, HDC, CE
Surface chemistry, coating composition, # of proton exchanging surface sites	TEM, SP-ICP-MS, XAS, Acid–Base Titrations

AAS-GF - Grafite Furnace Atomic Absorption Spectroscopy; AF4 - Assymetric Flow Field Flow Fractionation; AFM - Atomic Force Microscopy; CE - Capillary Electrophoresis; DLS - Dynamic Light Scattering; EDX - Energy Dispersive X-Ray; ESEM - Environmental Scanning Electron Microscopy; FESEM - Field-Emission Scanning Electron Microscopy; FFF – Field Flow Fractionation; FFF-ICP-MS - Flow-Field-Fractionation ICP-MS; HDC - Hydrodynamic Chromatography; HPLC - High Performance Liquid Chromatography; ICP-MS - Inductively Coupled Plasma-Mass Spectrometry; ICP-OES - Inductively Coupled Plasma-Optical Emission Spectrometry; IR - Infrared Spectroscopy; LC-ESMS - Liquid Chromatography-Electrospray Mass Spectrometry; LLEC - Liquid–Liquid Extraction Chromatography; MS – Mass Spectrometry; NMR - Nuclear Magnetic Resonance Spectroscopy; NTA - Nanoparticle Tracking Analysis; PCC - Particle Collision Coulometry; SAED - Selected area electron diffraction; Sd-FFF - Sedimentation-FFF; SP-ICP-MS - Single Particle ICP-MS; STEM – Scanning Transmission Electron Microscopy; TEM - Transmission Electron Microscopy; UV-vis - Ultraviolet–Visible Spectroscopy; VIP - Voltammetry of immobilized Particles; XAS - X-Ray Absorption Spectroscopy; XRD - X-Ray Diffraction

Environmental Hazard of Nanomaterials

With the development of nanotechnology over the past decade and a half, the number of reported NM effects in environmental species has grown exponentially. Based on the data available, effects have been classified for the variety of characteristics amongst the several NM tested (Bates et al., 2016). However, no full classification of effects has been done, considering the whole data available for NMs.

Comparing with the number of reports on aquatic species, much less is known for soil organisms (Klaine et al., 2008; Tourinho et al., 2012).

With the number of NMs available increasing with a multitude of characteristics of NMs being able to be altered and therefore changing the NM activity, the knowledge gap on NM toxicity will just increase and must be challenged.

Most of data on NMs and soil organisms is from population-level endpoints (survival, reproduction, growth), based on the standardized and well-defined existing protocols. While important for hazard assessment, there is a requirement to optimize/adapt these protocols to cover specific NM-related issues, with several work groups focusing on this matter (Kühnel and Nickel, 2014; Hund-Rinke et al., 2016; OECD, 2017).

The effect on population-level endpoints is normally a consequence of cumulative responses in the organisms making up such populations, starting from a very basic molecular level to a cellular level, affecting the physiological functions of the individuals and finally the species population. As such, assessment of changes on a cellular level can be used to predict or understand effects on higher levels, providing a useful tool in risk assessment.

Currently the standardized protocols for soil organisms used for testing NMs do not consider the dynamics that occur within an ecosystem: between the existing species and the surrounding environment. While these protocols may produce precise data in a laboratory-controlled environment, most focus only on one model organism. Therefore, are not designed to assess the route and interactions of NMs with the environment and other existing species. This has implications on the actual impact of the NMs to any ecosystem in Nature and it is an issue that must be addressed (Scott-Fordsmand et al., 2017).

Time has a relevant role for NM risk assessment as the human activity residues have large staying times in the soil and the interactions of NMs with the environment and the species on the long run are not known. Standardized tests for soil invertebrates comparing nano and non-nano forms of the same contaminant have produced different reports on the toxicity of different NMs, depending on the species and the time length of the testing (Amorim and Scott-Fordsmand, 2012; Gomes et al., 2013; Schlich et al., 2013; Waalewijn-Kool et al., 2013; Noordhoek et al., 2018), while short-term exposures do not fully show the toxicity potential of NMs (Diez-Ortiz et al., 2015). On the other hand, longer-term exposure has been shown to produce higher effects due to nano than salt form (Gonçalves et al., 2017). As such, the dependency of time should be further studied to fully assess the response of soil invertebrates to NMs

Summarizing, testing NMs in increasingly complex exposure media, including the development of small scaled food-webs, and extending the current timeframe of the tests, both for the same population or several generations of the species, will produce more realistically accurate data (Baalousha et al., 2016). On this matter, several tests have been developed over the past decade, some being adaptations of current standard test guidelines that have not yet been used with NMs but can bridge the gap: multigenerational tests (Lock and Janssen, 2002; Campiche et al., 2007; Paumen et al., 2008; Amorim et al., 2017; van Gestel et al., 2017) and soil multispecies system tests (Scott-Fordsmand et al., 2008; Jensen and Scott-Fordsmand, 2012; Menezes-Oliveira et al., 2013, 2014; Schnug et al., 2014).

Test Organisms and Endpoints

Test Organisms

Responses to NMs are species specific and can have a larger range of differences. This has an impact on the effect of NMs in ecosystems due to the existence of several species and their interactions.

Soil invertebrate systems are relevant in NM risk assessment due to their low positioning in food-webs and involvement in soil functioning and consequently have a supporting role in the ecosystem, being easily exposed and affected by soil toxicants (Pey et al., 2014). Here are presented representative species with different functions in a soil invertebrate ecosystem, representing different subclasses: one oligochaete, one acari and four collembolans (Figure 1).

Figure 1: Species representative of a soil invertebrate ecosystem. A: *Enchytraeus crypticus*; B: *Hypoaspis aculeifer*; C: *Folsomia candida*; D: *Proisotoma minuta*; E: *Hypogastrura assimilis*; F: *Mesophorura macrochaeta*.

Enchytraeus crypticus (Annelida: Oligochaeta: Enchytraeid) (Westheide and Graefe, 1992) is a soil dwelling species. The enchytraeid species degrade organic matter and improve the pore structure of the soil (Amorim et al., 2005b). It has been used as a model organism for terrestrial ecotoxicological studies (Castro-Ferreira et al., 2012) and included in standardized protocols (OECD, 2004).

Hypoaspis aculeifer (Acari: Laelapidae) is a relevant representative of predatory mites and it can be found worldwide. The species' life history has been thoroughly described (Lesna et al., 1996; Hamers and Krogh, 1997; Berndt et al., 2003; Baatrup et al., 2006; Heckmann et al., 2007; Pfeffer and Filser, 2010; Owojori et al., 2013; Huguier et al., 2015) and is used as a model organism in standardized protocols (OECD, 2008). Its addition as part of soil multispecies system (Heckmann et al., 2007; Scott-Fordsmand et al., 2008) allows the assessment of the influence of a biological stressor species into NM toxicity on other species in the study.

The collembolan species are representative of the most abundant (micro) arthropods, being sensitive to soil contamination and widely used in assessment of environmental risk (Fountain and Hopkin, 2005). Amongst the collembolan species used we highlight the family *Isotomidae* (*Folsomia candida* and *Proisotoma minuta*), *Hypogastruridae* (*Hypogastrura assimilis*) and *Onychiuroidea* (*Mesophorura macrochaeta*) (Fjellberg, 1998). In the soil ecosystem, collembolans are known to be grazers and act as leaf decomposer, mainly feed on bacteria and fungi (Fountain and Hopkin, 2005), potentially developing a competitive interaction for food source with enchytraeids, while representing different routes for contaminant exposure. They are frequently preyed on by other species, namely *H. aculeifer* and as such, are also used as food source for acari standard testing (OECD, 2008). *F. candida* is used as a model organism in standardized protocols for soil contamination due to its high reproductive rate and relative sensitivity to chemicals, being easily bred in laboratory conditions (OECD,

2009). Other collembolan species such as *M. macrochaeta* (Niklasson et al., 2000), *H. assimilis* (Amorim et al., 2005a) and *P. minuta* (Nursita et al., 2005, 2009; Park and Lees, 2006; Buch et al., 2016) have been thoroughly studied or use for soil contamination studies.

Collembolans feed on soil for energy and directly uptake it into the ventral tube (Hopkin, 1997; Fountain and Hopkin, 2005), while contaminants can become attached to their cuticle and enter the organism through the cuticle pore canals (Hopkin, 1997; Fountain and Hopkin, 2001). After the moulting process, collembolans can feed on their own former exoskeleton, possible digesting any trace of contaminants attached to it (Hopkin, 1997).

Being part of the same subclass, the collembolan species interactions within the ecosystem, between themselves and other species, particularly predators such as *H. aculeifer*, result in a food-web that can be used as a tool to predict the response to a stressor.

Species interaction and its consequences (extinction, fitness of the population) are predicted on the data obtained from soil multispecies system tests (Scott-Fordsmand et al., 2008; Menezes-Oliveira et al., 2013, 2014) which can be used to assess the toxic effects of NMs in an exposure media with higher complexity than standard testing, in a way more similar to a terrestrial ecosystem.

Biological and Populational Endpoints

As mentioned before, an effect on a biological/organism level is likely a consequence of changes at lower levels of organization, i.e. cellular level. On the other hand, effects on a population level are result of changes to the individuals, which, depending on the social behaviour of each species, may result in extinction or expansion. The fact that species interact will consequently have an impact on a higher level, such as community and the whole ecosystem (Figure 2).

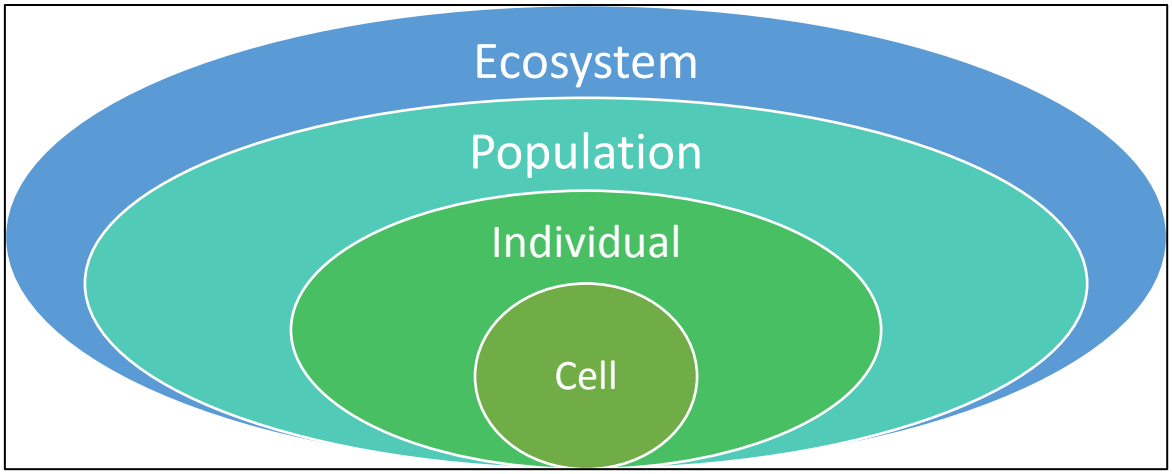

Figure 2: Schematic representation of the different organizational levels in study, with the cellular level as the support. The higher levels of organization result in increasingly complexity media for NM exposure.

The endpoints of interest in this study are described next:

Cellular Level: Biochemical Responses - Oxidative Stress Parameters

Oxidative stress refers to a disturbance in the pro oxidant-antioxidant balance in favour of the pro-oxidants, leading to the formation of reactive oxygen species (ROS) and potential damage. ROS are partially reduced forms of molecular oxygen (O_2) which are normal products of cellular metabolism and can also be produced by cells as a protective mechanism or in response to stress (e.g. xenobiotics). Normal cells can remove ROS by antioxidant defences, however larger increases in ROS production can unbalance the equilibrium between pro-oxidants and antioxidant defences leading to oxidative stress (Andrews, 2000).

The antioxidant defence mechanism is comprised of enzymatic and non-enzymatic antioxidants. The enzymatic antixoxidants include SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase) and GSTs (glutathione-S-transferases), while in non-enzymatic defences there is gluthatione and the induction of metallothionein.

SOD acts by converting O_2^- into H_2O_2 which is then detoxified by CAT and GPx. Detoxifying by GPx involves the use of GSH (reduced glutathione) as reducing agent which is oxidized to GSSG; the GPx cycle is closed by the re-conversion of GSSG into GSH by GR (glutathione reductase). GSTs can catalyse the conjugation of GSH with several xenobiotics. Besides the GSH, glutathione can also act by linking directly to pro-oxidants (like transition metals or sulphidryl groups), by conjugating electrophilic

xenobiotics during GST activities and by participating in the regeneration of other antioxidants (i.e. ascobate and tocopherol) (Filomeni et al., 2002)

Metallothioneins (MTs) are metal binding proteins which protect cells from metal toxicity by acting as chelating agent of the excess of toxic metals, and also provide protection against oxidative stress as effective scavenger of hydroxyl radicals (Maria et al., 2014).

Lipid peroxidation products, i.e. Malondialdehyde, are representative of oxidative damage, resulting from the release of ROS, indicating a higher antioxidant defence activation in response to a stressor (Zhang et al., 2012).

An overview of the antioxidant defences can be observed in Figure 3.

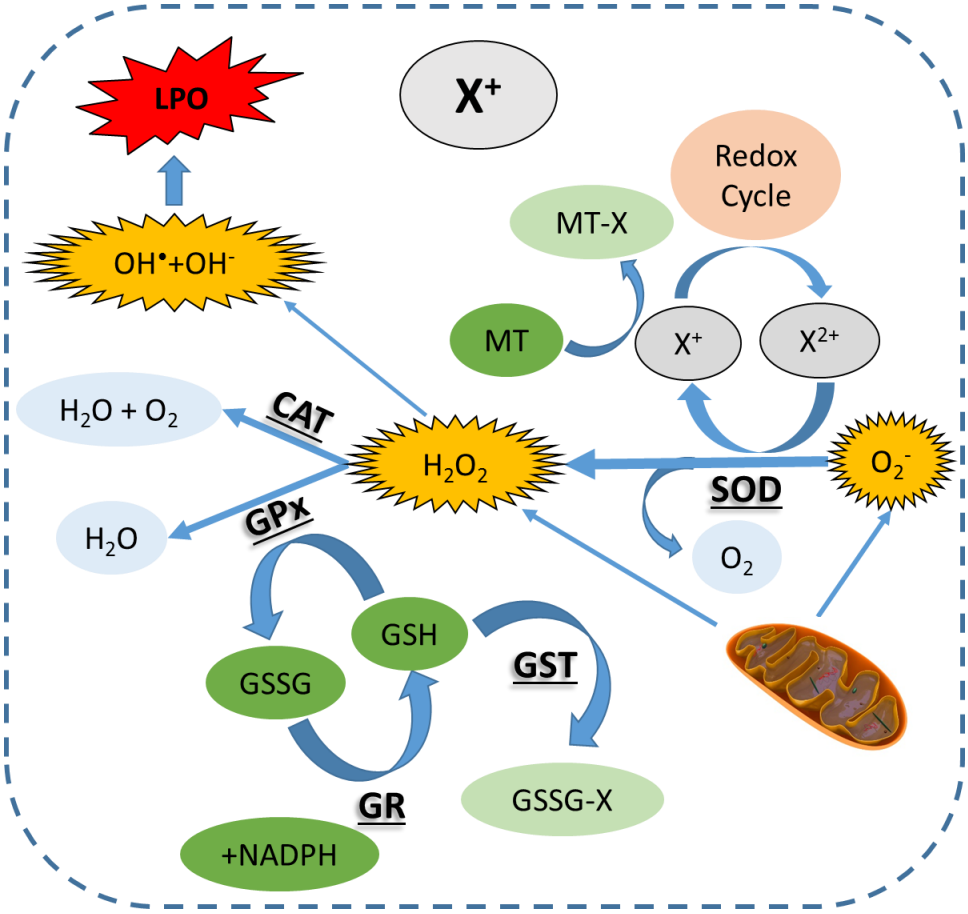

Figure 3: Schematic representation of the cellular antioxidant defence mechanisms in the presence of a generalist oxidative stressor X.

Cellular Level: Metal Internal Concentration

Due to soil grazing and feeding on fungi and bacteria attached to the soil, collembolans can uptake metals that are present in the soil constitution. In soils with high concentration of metals this is reflected in the internal concentration of these metals (Pedersen et al., 1997, 1999; Waalewijn-Kool et al., 2014). The excessive concentration of metals can result in the development of oxidative stress and therefore having an impact on cellular and consequently organism level. Beside the antioxidant defences, collembolans possess mechanisms for metal excretion activated once a threshold of metal internal concentration is reached (Ardestani et al., 2014).

Considering the use of metal-based NMs and the occurrence of transformation such as dissolution and release of metals in ionic forms (Lowry et al., 2012), the quantification of metal in the organism allows a prediction on the level of toxicity due to NM exposure (Kittler et al., 2010; Waalewijn-Kool et al., 2013; Cornelis et al., 2012).

Population Level: Survival/Reproduction/Predation/Abundance

Standard ecotoxicity guidelines have been developed or under development by the International Organization for Standardization (ISO) and by the Organization for Economic Cooperation and Development (OECD) to assess survival and/or reproduction of collembolans (OECD, 2009; ISO, 2014), enchytraeids (ISO, 2004; OECD, 2004) and mites (OECD, 2008).

These tests range from acute (assessment of survival, in short periods of exposure to highly toxic concentrations) to chronic (e.g. assessment of reproduction, in longer exposure periods to sub-lethal concentrations).

The predatory behaviour is dependent on the species, both the predator and the preys available. While predation protocols have not been standardized yet, it has an important role in ecotoxicological studies (Huguier et al., 2015). It is considered in protocols for reproduction and survival of predatory mites (*H. aculeifer*) (OECD, 2008) and have an impact on the reproductive output of the species (Baatrup et al., 2006; Hamers and Krogh, 1997). Predation can also be influenced by contaminant exposure (Madani et al., 2015; Owojori et al., 2013), life stages (both prey and predator) and the variety of prey available, namely their biomass (Heckmann et al., 2007; Sabelis and Lesna, 2010).

Predation affects species' population size in an ecosystem (Lesna et al., 1996) and the interactions between the remaining species and their surroundings in response to pollutants (Cortet et al., 2006).

Because of survival, reproduction and predation by other species, the resulting species abundance is the total number of individuals in all life stages of a species. In an ecosystem the total abundance may be stable, but each species abundance may vary with time. These dynamics result from the response to a stressor together with species-interactions (Figure 4).

These parameters are useful tools in assessing the effects of NMs on the species making up the community.

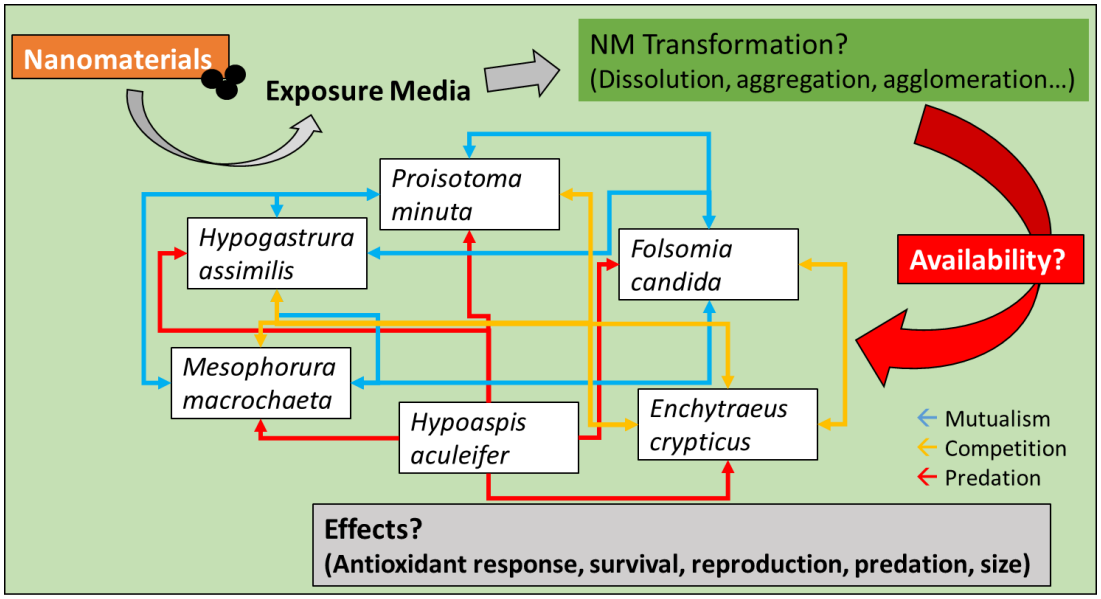

Figure 4: Schematic representation of the NMs fate on a complex exposure media like an ecosystem, with transformation in the exposure media affecting the NM availability to species community. Species interactions also affect the NM availability for each species and reciprocally. The effects due to NM exposure can be measured by the mentioned parameters.

Aim and Outline of the Thesis

Considering the increasing number of NMs becoming available and their potential impact, the aim of this thesis was to assess the effect of selected NMs, after exposure to increasingly complex scenarios, on *F. candida* (as a model organism) on different organizational levels (cellular, organism, populational and ecosystem) and set relationship between the observed effects, with the data and information to be used in

environmental risk assessments. The different scenarios were developed considering the following variables as contributing to the effect of nanomaterials:

1. Time: using different testing time intervals ranging from 10 days to 186 days
2. Potential transformations and compartmentalization of the NMs in the exposure media (soil, soil-solution), using salt form as reference for comparison of ion release.
3. Species interactions, i.e. predation, as a magnifying factor to exposure.
4. Modification of NM surface (addition of coatings) that impact the NM interactions with the media and organisms.

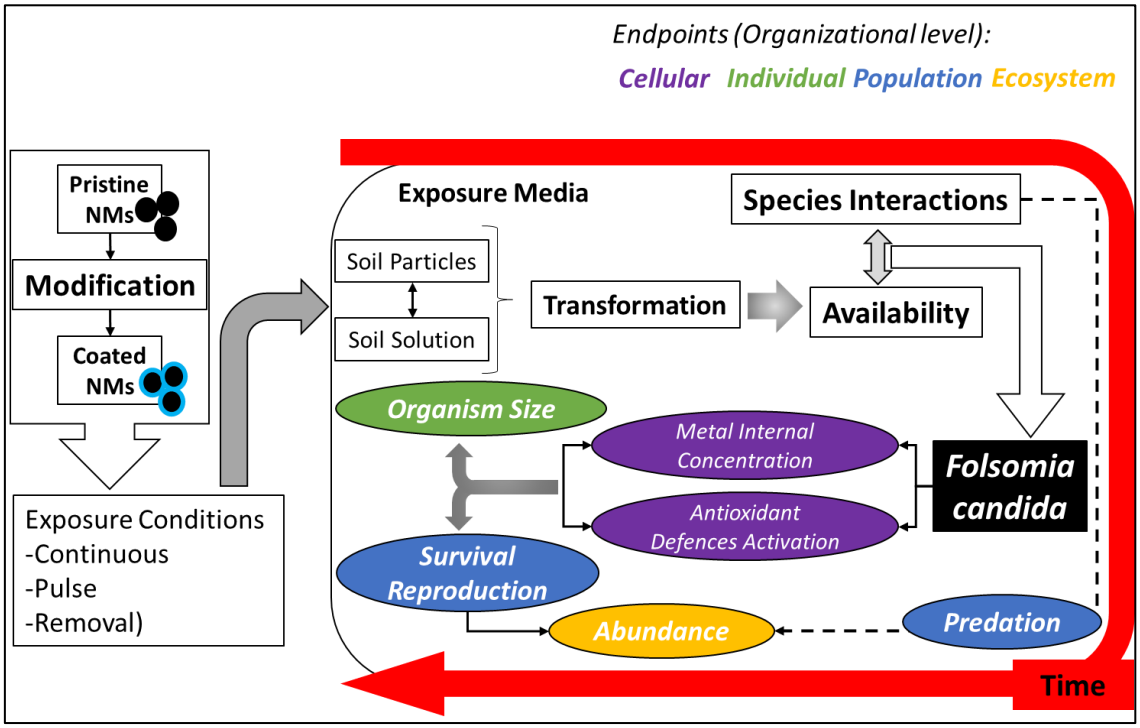

Figure 5: Schematic overview of the thesis, highlighting the variables that affect the effect of NMs (Time; Transformation; Species Interactions; Modification) and the endpoints used at different organizational levels.

The results are organized in four chapters (chapter two to five), in the format of peer-reviewed journal publications, and an integrated overview of the results (chapter six), as follows:

C**hapter two:**

Ag Nanoparticles (Ag NM300K) in the Terrestrial Environment: Effects at Population and Cellular Level in Folsomia candida (Collembola)

Mendes L.A., Maria V.L, Scott-Fordsmand J.J., Amorim M.J.B.

International Journal of Environmental Research and Public Health (2015), 12(10), 12530-12542

DOI:10.3390/ijerph121012530 (Mendes et al., 2015)

In this chapter the impact of silver NMs on *Folsomia candida* on a short-term and standardized timeframe were assessed, evaluated on two organization levels: cellular (antioxidant response) and populational (survival and reproduction) and the relationship of a cause-consequence between the two being established. The effects were compared to the ones resulting from silver salt form exposure to assess the contribution of time to the development of toxicity from NMs versus the active form of silver. In this chapter it was shown that both the salt and the nano form resulted in responses on a cellular level, with salt having an earlier response than nano which resulted on effects on a populational level that were only observed for the salt form in the standardized test timeframe.

Chapter three:

Multigenerational Exposure of Folsomia candida to Silver: Effect of Different Contamination Scenarios (Continuous versus Pulsed and Recovery)

Mendes L.A., Maria V.L, Scott-Fordsmand J.J., Amorim M.J.B.

Science of The Total Environment (2018), 631-32, 326-333

DOI: 10.1016/j.scitotenv.2018.02.332 (Mendes et al., 2018a)

Based on the results from chapter two, the effects because of time of the non-nano form of silver were assessed in a long-term multigenerational study in chapter three. In this, potential transgenerational effects, population extinction or recovery after the exposure were evaluated in different exposure patterns. A relationship between individual level endpoints (size) and population level (survival and reproduction) in response to the exposure was developed. The reproductive output was impaired once the organisms

were exposed for at least one generation to silver and did not fully recover in clean soil. While the decrease in reproduction was observed in the generation exposed to silver, the decrease in the size of the organisms was only observed after three generations, regardless of the exposure pattern, reflecting the development of transgenerational effects due to the initial exposure to silver.

Chapter four:

Interactions of Soil Species Exposed to CuO NMs are Different from Cu Salt – A Multispecies Test

Mendes L.A., Amorim M.J.B., Scott-Fordsmand J.J.

Environmental Science & Technology (2018); 52 (7), 4413-4421

DOI: 10.1021/acs.est.8b00535 (Mendes et al., 2018b),

In this chapter the presence of other species and the interactions amongst them, e.g. predation, as a contributing factor for NM effect was studied. This allowed to study the impact of NMs and the dynamics of an ecosystem overtime. Also, in this study the fate of NMs in the exposure media with time was evaluated by quantifying the total and active metal form concentration in the soil, soil solution and animals. A multi-endpoint approach between cellular (metal internal concentration), population (survival/reproduction/predation) and ecosystem (abundance) endpoints allowed to compare the long-term effects of CuO NMs and the salt counterpart ($CuCl_2$). While salt exposure resulted in a more immediate response, the toxicity because of NM exposure increased with time. The species sensitivity to NM exposure was higher in the multispecies system than in individual testing, with predation and NM exposure having a synergistic effect.

Chapter five:

Zeta potential to predict nanomaterials toxicity and safety by design - on surface modified CuO NMs using a soil multispecies test system

Mendes L.A., Amorim M.J.B., Scott-Fordsmand J.J.

submitted

Based on chapter four, chapter five focused on the effect of the modification of pristine NMs to nanotoxicity. Four different coated from the same pristine NM were developed and exposed to a multispecies system, following a similar multi-endpoint approach on cellular, population and ecosystem levels, as in chapter four. This allowed to evaluate how the modification of NM characteristics would alter the fate of NMs on exposure media, the availability to the organisms and the toxicity, reflecting a realistic scenario of exposure. From this, it was observed that the acquired zeta potential induced different responses for each species, affecting the species interactions, i.e. predation, and the ecosystem composition and dynamics on the long term. Positive polymeric coatings were less toxic than negative charged coatings while non-charged NMs resulted in a more immediate toxic effect.

In **Chapter six: General Discussion and Conclusions**, the results in the preceding chapters are discussed in an integrated perspective, highlighting the main conclusions derived from this work.

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Chapter two

Ag Nanoparticles (Ag NM300K) in the Terrestrial Environment: Effects at Population and Cellular Level in *Folsomia candida* (Collembola)

Ag Nanoparticles (Ag NM300K) in the Terrestrial Environment: Effects at Population and Cellular Level in *Folsomia candida* (Collembola)

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Abstract

The effects of nanomaterials have been primarily assessed based on standard ecotoxicity guidelines. However, by adapting alternative measures the information gained could be enhanced considerably, e.g., studies should focus on more mechanistic approaches. Here, the environmental risk posed by the presence of silver nanoparticles (Ag NM300K) in soil was investigated, anchoring population and cellular level effects, *i.e.*, survival, reproduction (28 days) and oxidative stress markers (0, 2, 4, 6, 10 days). The standard species *Folsomia candida* was used. Measured markers included catalase (CAT), glutathione reductase (GR), glutathione S-transferase (GST), total glutathione (TG), metallothionein (MT) and lipid peroxidation (LPO). Results showed that $AgNO_3$ was more toxic than AgNPs at the population level: reproduction EC_{20} and EC_{50} was ca. 2 and 4 times lower, respectively. At the cellular level Correspondence Analysis showed a clear separation between $AgNO_3$ and AgNP throughout time. Results showed differences in the mechanisms, indicating a combined effect of released Ag^+ (MT and GST) and of AgNPs (CAT, GR, TG, LPO). Hence, clear advantages from mechanistic approaches are shown, but also that time is of importance when measuring such responses.

Keywords: antioxidant defenses; mechanisms of response; soil invertebrates.

1. Introduction

The effects of nanomaterials have been primarily assessed via the use of standard ecotoxicity guidelines, although, there are evidences that adaptations and alternatives should be considered, e.g., the required exposure time should be adjusted (Gomes et al., 2015). The use of more mechanistic based studies can provide many advantages supporting the present standard tests, e.g., understanding the mode of action can be used as a background for extrapolating from short to long-term effects, an issue which has high priority (Scott-Fordsmand et al., 2014).

Silver nanoparticles (AgNPs), which are widely used due to their bactericidal properties, have also been reported toxic for a diverse range of organisms, e.g., for soil invertebrates such as *Eisenia fetida* (Gomes et al., 2015; Hayashi et al., 2012, 2013a), *Enchytraeus albidus* (Gomes et al., 2013), *Eisenia andrei* (Schlich et al., 2013), *Porcellio scaber* (Tkalec et al., 2011) and *Folsomia candida* (Waalewijn-Kool et al., 2014). One of the known mechanisms related to Ag toxicity is the induction of oxidative stress. This process is the result of the increase of reactive oxygen species (ROS) in the organism, causing an unbalance and activation of the antioxidant defense mechanisms (Andrews, 2000; Valavanidis et al., 2006). These include the activation of several enzymatic and non-enzymatic proteins, such as catalase (CAT), glutathione reductase (GR) or metallothioneins (MTs). The methodology to measure such markers has been optimized for various soil organisms, including *Folsomia candida* (Maria et al., 2014), *Enchytraeus albidus* (Gomes et al., 2012) or *Eisenia fetida* (Gomes et al., 2015).

Here, the environmental effect of silver nanoparticles (Ag NM300K) in soil was investigated, anchoring population and cellular level effects, *i.e.*, survival, reproduction (standard test, 28 days) after which the oxidative stress markers were evaluated at the reproduction Effect Concentration that Causes 50% Reduction (EC_{50}), along an exposure time series: 0, 2, 4, 6, 10 days. The species *Folsomia candida* (Collembola) was used as test species. Collembolans have been widely used to assess the environmental impact of e.g., organic chemicals (Scott-Fordsmand and Krogh, 2004), pesticides (Amorim et al., 2005), metals (Nakamori et al., 2010), mixtures (Amorim et al., 2012) or nanomaterials (Waalewijn-Kool et al., 2013a, 2013b, 2014). The markers used were catalase (CAT), glutathione reductase (GR), glutathione S-transferase (GST), total glutathione (TG), metallothionein (MT) and lipid peroxidation (LPO).

2. Material and Methods

2.1. Test Organism

Folsomia candida (Collembola) was used as test species (ISO, 2014). Cultures were maintained in laboratory on a moist substrate of Paris plaster and activated charcoal (8:1 ratio) at 19 ± 1 °C, under a photoperiod regime of 16:8 (light:dark). The organisms were fed once a week with dried baker's yeast (*Saccharomyces cerevisae*). Organisms of synchronized age (10–12 days) were used for the experiments, as within the standard protocol.

2.2. Test Materials

Test materials included Ag salt and Ag nanomaterial. The $AgNO_3$ (high-grade, 98.5%–99.9% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The silver nanoparticles (AgNPs) used were the standard reference materials Ag NM300K from the European Commission Joint Research Centre (JRC), fully characterized (Klein et al., 2011). The Ag NM300K is dispersed in 4% polyoxyethylene glycerol triolaete and polyoxyethylene (20) sorbitan monolaurate (Tween 20), thus the dispersant was also tested alone.

2.3. Test Soil and Spiking

Natural standard from the "Landesanstalt für Umwelt und Forschung"(LUFA) soil 2.2 (Speyer, Germany) was used. The general soil properties are as follow: pH = 5.5, organic carbon = 1.77%, cation exchange capacity = 10.1 meg/100 g, and grain size distribution of 7.3% clay; 13.8% silt and 78.9% sand.

Ag was spiked as aqueous solution and serially diluted. The soil was pre-moistened before spiking, to obtain a final water holding capacity of 50%, and aged for 72 h before test start. For Ag NM300K, spiking was done individually for each replicate. For $AgNO_3$, the various replicates per treatment were spiked together and then divided into each test vessel as within standard. Concentration range for $AgNO_3$ was: 0, 64, 100, 130, 320, 640 mg Ag/kg soil dry weight (DW) and for AgNP was: 0, 64, 130, 220, 320, 640 mg Ag/kg soil DW. A control dispersant was used adding the same volume as used with the highest concentration of Ag NM300K to assess the effect of the dispersant alone. Test concentration used for the biomarker exposure corresponded to the reproduction EC_{50} (value selected within the confidence interval). The choice of this EC50 was based on its relevance in Risk

Assessment and linkage to reproduction chronic effects. Moreover, the tested concentration should be sub-lethal to ensure organisms' survival for sampling and for mechanistic studies before narcosis (not relevant for biomarkers).

2.4. Test Procedure

2.4.1. Population Level—Standard Reproduction Test

Tests followed the standard reproduction of International Organization for Standardization (ISO) test guideline for collembolans (ISO, 2014). In short, 10 juveniles (10–12 days) were transferred to the test vessels containing the soil. Four replicates were used per treatment. Test ran at 20 °C and 16:8 h (light:dark) photoperiod; food supply and water was replenished every week. Reproduction and adult survival were assessed after 28 days by flotation method to count the number of adults and juveniles.

2.4.2. Cellular Level—Oxidative Stress Biomarkers

Procedures followed the same as in the standard guideline (ISO, 2014) with adaptations (Maria et al., 2014). A pool of 50 juveniles of 13–14 days was used as a replicate. Ten (10) replicates (five for MT measurements plus five for the other markers) per treatment were performed. Exposure period included samplings at 0, 2, 4, 6, 10 days. At each sampling time organisms were extracted by flotation, transferred to plaster to absorb the excess water and pooled into microtubes, weighted and snap-frozen in liquid nitrogen, being stored at −80°C until further analysis. Five replicates per condition were used for metallothionein (MT) quantification and the other five for the rest of all biochemical analysis, *i.e.*, catalase (CAT), glutathione reductase (GR), glutathione S-transferase (GST), total glutathione (TG) and lipid peroxidation (LPO). Biomarkers measurements were performed following the procedures as described in Maria *et al.* (2014).

2.5. Data Analysis

One-way ANOVA and Post Hoc Dunnett's test was used to identify significant differences between control and treatments (Sigmaplot, 1997). The effect concentrations (ECx) were calculated using the Toxicity Relationship Analysis Program (TRAP 1.21) applying the 2-parameters Logistic model. To assess differences between control and control dispersant a t-test ($p < 0.05$) was used.

Multivariate analysis was done using Correspondence Analysis (CA) including all treatments.

The analysis was performed using the software SAS Enterprise Guide 5.1 (*SAS Enterprise Guide 5.1*, 2012). To compensate for the different scales of the biomarkers, the response was normalised before use, several different normalisation methods were tested overall giving the same pattern; the present normalisation was based on averaging in relation to the mean.

3. Results

3.1. Materials Characterization

The silver nanoparticles (AgNPs) used were the standard reference materials Ag NM300K from the European Commission Joint Research Centre (JRC), fully characterized (Klein et al., 2011). In short, Ag NM300K are spherical and consist of a colloidal dispersion with a nominal silver content of 10.2 w/w %, dispersed in 4% w/w of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan monolaurate (Tween 20), having > 99% number of particles with a nominal size of about 15 nm, with no coating. Transmisson Electron Microscopy (TEM) indicated a size of 17 ± 8 nm. Smaller nanoparticles of about 5 nm are also present.

3.2. Biological Characterization

3.2.1. Population Level—Standard Reproduction Test

The tests validity criteria were fulfilled, *i.e.*, less than 20% mortality, >100 juveniles per replicate and the coefficient of variation < 30%. Results can be observed in Figure 1. For Ag NM300K no differences between control and control dispersant were observed ($p > 0.05$): Adult survival (average (AV) ± standard error (SE)): 10 ± 0; Juvenile Reproduction (AV±SE): 977 ± 50 and 1004 ± 47, respectively, hence data was modeled pooling both controls. A dose response effect was observed, with Ag NM300K being less toxic than $AgNO_3$. The estimated ECx values can be seen in Table 1.

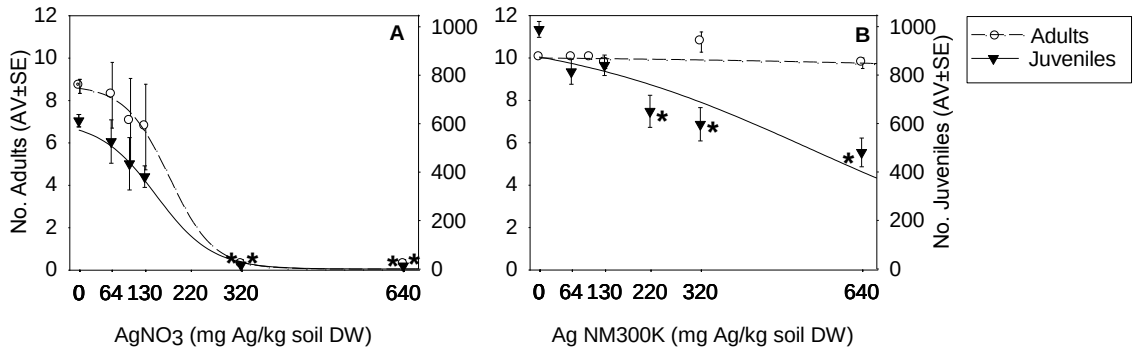

Figure 1. Survival (number of adults) and reproduction (number of juveniles) for *Folsomia candida* when exposed in LUFA 2.2 soil to (**A**) $AgNO_3$ and (**B**) Ag NM300K. Results are expressed as average ± standard error (Av ± SE) (n = 4). *****: Dunnett's ($p < 0.05$) for differences between control and treatments. Lines represent the model fit to data.

Table 1. Effect Concentrations (ECx) for survival and reproduction of *Folsomia candida* when exposed to $AgNO_3$ and AgNPs (Ag NM300K). n.d.: not determined. n.e.: no effect (95% Confidence Intervals). $EC_{10, 20, 50, 80}$: Concentration that causes 10%, 20%, 50%, 80% Effect, respectively. S: relative slope estimated at EC_{50}, Y0: Average control value (average of control values for survival and reproduction).

materials	**EC_{10} (mg/kg)**	**EC_{20} (mg/kg)**	**EC_{50} (mg/kg)**	**EC_{80} (mg/kg)**	**Model and Parameters**
			Survival		
$AgNO_3$	82 (20–162)	118 (62–174)	179 (77–280)	240 (57–422)	Logistic 2 parameters (S:0.0057; Y0:8.7)
Ag NM300K	n.e.	n.e.	n.e.	n.e.	–
			Reproduction		
$AgNO_3$	31 (−35–97)	76 (36–115)	152 (108–196)	228 (134–324)	Logistic 2 parameters (S:0.0045; Y0:610.0)
Ag NM300K	n.d.	173 (70–277)	540 (412–667)	906 (653–1159)	Logistic 2 parameters (S:0.0009; Y0:988.3)

3.3. Cellular Level—Oxidative Stress Biomarkers

3.3.1. Univariate Analysis

For Ag NM300K the control dispersant was used as a reference because for LPO, TG and GR measurements there were differences ($p < 0.05$) between control and control dispersant (Figure 2).

For $AgNO_3$, CAT activity decreased after 4 days exposure ($p < 0.05$) (0.4-fold to control), maintaining a tendency of low values in the remaining exposure time. GR activity shows an increase after 2 and 4 days ($p < 0.05$) followed by a decrease to levels lower than control at 6 and 10 days exposure. MT shows a similar pattern. GST activity shows an increase-decrease-increase behaviour at 4 ($p < 0.05$), 6 and 10 ($p < 0.05$) days respectively and TG increased only at day 4 ($p < 0.05$). Significant increase in LPO levels was observed at day 4 (1.2-fold, $p < 0.05$).

For Ag NM300K CAT activity was higher after 2, 4 and 10 days ($p < 0.05$), having a decrease tendency at day 6 (to levels similar to control). GST activity showed an increase ($p < 0.05$) up to day 4 and then continued on same levels until day 10 ($p < 0.05$). GR increased only after 4 days ($p < 0.05$). TG levels were lower than control after 4 days and superior at 10 days. MT levels increased after 6 days ($p < 0.05$), maintaining the higher level at day 10. LPO increased at day 2 ($p < 0.05$), after which it decreased to be increased again at day 10 ($p < 0.05$).

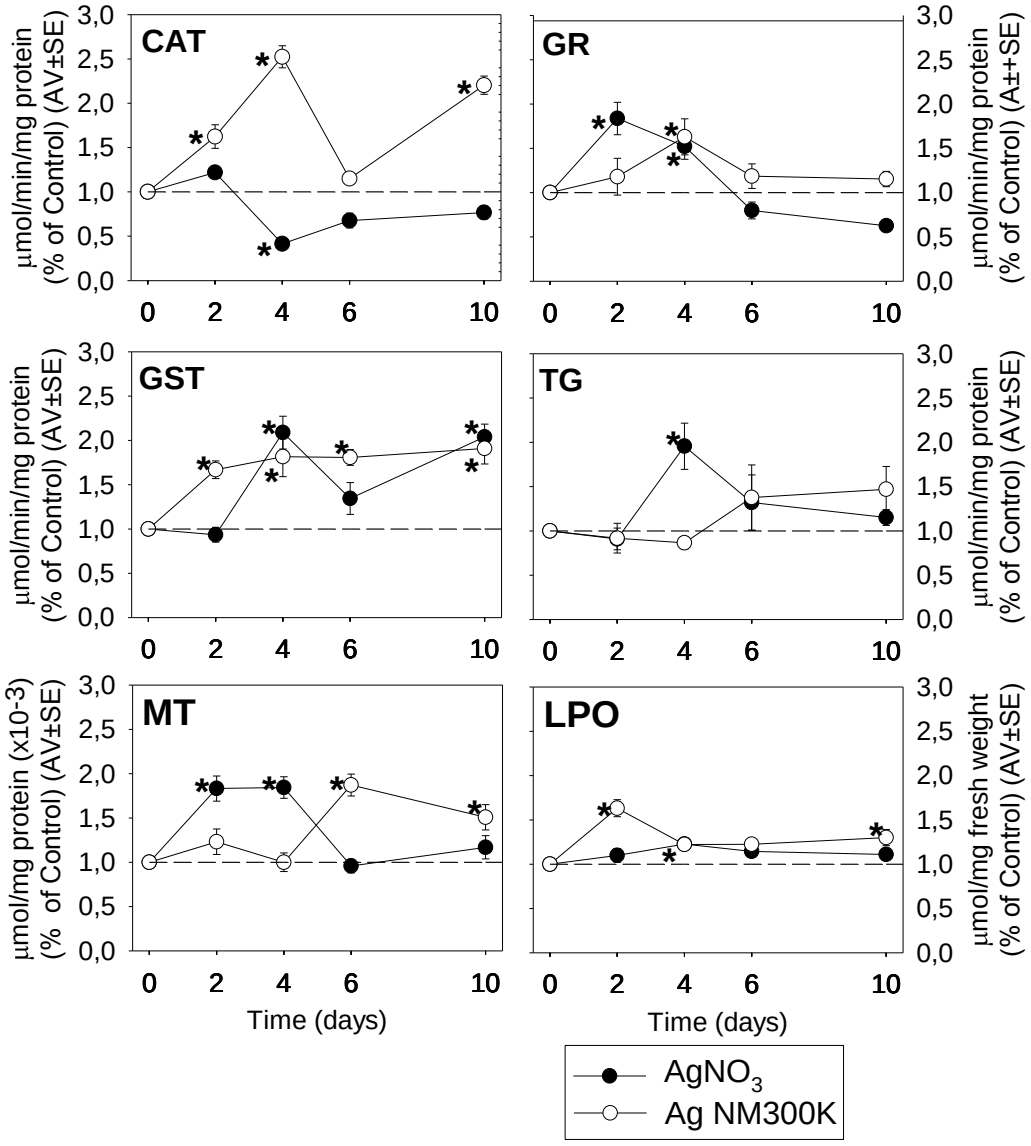

Figure 2. Oxidative stress biomarker results for *Folsomia candida* when exposed in LUFA 2.2 soil to the reproduction EC_{50} of $AgNO_3$ (black dots) and Ag NM 300K (white dots). Results are expressed as % and normalized to the respective controls (water and dispersant) mean values ± standard error (Av ± SE) (n = 5). Dotted horizontal line represents the control reference, *i.e.*, 100%. CAT: Catalase; GR: Glutathione Reductase (GR), GST: Glutathione S-Transferase, TG: Total Glutathione; MT: Metallothionein; LPO: Lipid Peroxidation; *****: Dunnett's test ($p < 0.05$) for differences between control and treatments.

3.3.2. Multivariate Analysis

The multivariate analysis of the data (Correspondence Analysis) enabled an identification of the overall differences between the $AgNO_3$ and AgNP exposures (Figure 3), with a mainly clear separation between the $AgNO_3$ and AgNP throughout time. [It should be noted that whereas Figure 2 shows mean values and standard errors,

the multivariate plot displays the individual replicates]. It is seen that LPO and GST were primarily associated with AgNP and MT and TG associated with $AgNO_3$, hence these markers would be the primary identifiers of different exposures. In the later exposure stages (10 days) the GR was most pronounced for the AgNP exposure, when compared to $AgNO_3$ exposure. The larger confidence ellipse (compared to others) related to the $AgNO_3$ at day 6, seems to be related to one replicate having a relative high (again compared to the others) TG.

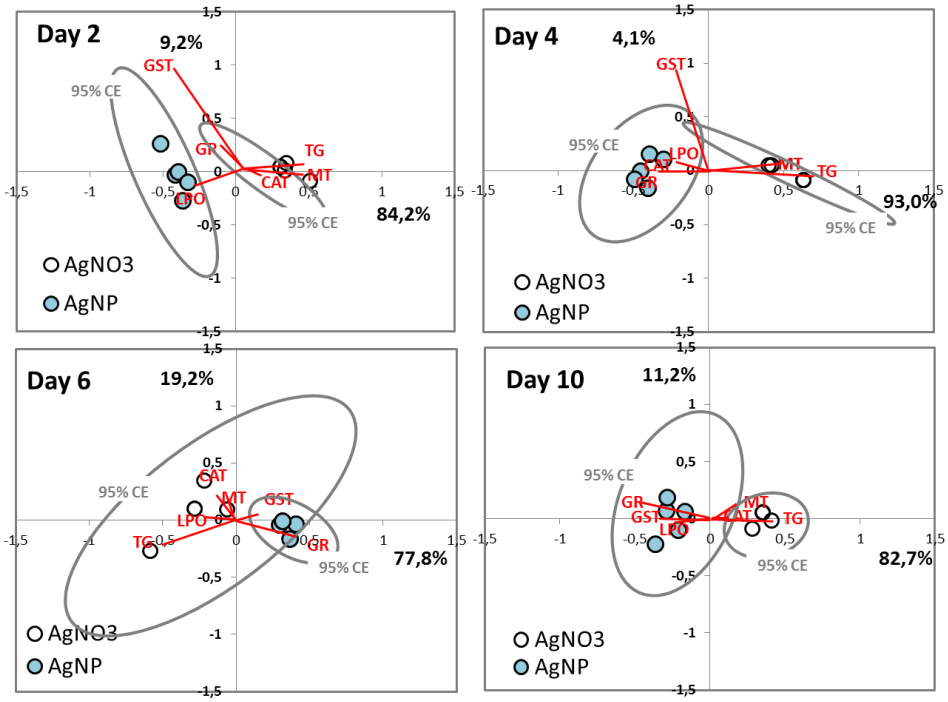

Figure 3. Correspondence Analysis (CA) of data from *Folsomia candida* exposed to AgNP (Ag NM300K) [640 mg Ag/kg soil] and $AgNO_3$ [145 mg Ag/kg soil], as sampled at 0-2-4-6-10 days, in terms of Catalase (CAT), Glutathione Peroxidase (GPx), Glutathione S-Transferase (GST), Glutathione Reductase (GR), Total Glutathione (TG), Metallothionein (MT) and Lipid Peroxidation (LPO). Percentage (%) explanatory power is added for each axis. All time points showed significant differences (discriminant analysis), the day 6 timepoint shows largest overlap of the two confidence ellipses, which show the difference here is the least.

4. Discussion

4.1. Population Level

Results showed that $AgNO_3$ displayed higher toxicity than Ag NM300K for *Folsomia candida*, with increasing difference with higher concentration (EC_{20} to EC_{80}). For $AgNO_3$, the Effect Concentration (EC) values were within the obtained confidence interval as found by Waalewijn-Kool *et al.* (2014) for *F. candida* tested under the same conditions. The same authors tested other AgNP (paraffin coated, 3–8 nm, water dispersed) and found no effect on survival or reproduction up to 673 mg Ag/kg soil DW. As concluded by the authors, the internal Ag concentrations for *F. candida* could not explain the higher toxicity of $AgNO_3$ compared to AgNPs; it has been suggested that the higher internal Ag in *F. candida* exposed to AgNPs could be because these are taken up on the particulate form. Unlike ZnO NPs (Kool et al., 2011; Waalewijn-Kool et al., 2013a), porewater concentrations could not explain the toxicity of AgNPs. It seems that AgNPs aggregation and sorption to soil parts reduces dissolution. The fate of AgNPs in soil has been reported complex, with e.g., soil type, dissolution (rate), oxidation, nanoparticle size and the type of coating influencing the availability of Ag (Waalewijn-Kool et al., 2014). For other invertebrates, oligochaete studies have shown that $AgNO_3$ was more toxic than AgNPs (Heckmann et al., 2010; Shoults-Wilson et al., 2011; Gomes et al., 2013; Schlich et al., 2013). Van der Ploeg *et al.* (2014) observed that low doses of the same Ag NM300K (15 mg Ag/kg soil DW) caused higher effects (for the same mass concentration) than $AgNO_3$ in *Lumbricus rubellus* longer term reproduction study. Moreover, also focusing on longer term exposures, Bicho *et al*.(2016) showed that in an *Enchytraeus crypticus* full life-cycle test 20 mg Ag/kg soil DW of Ag NM300K caused an effect equivalent to the reproduction EC50, although the dose response model estimated an EC_{50} = 80 mg Ag/kg soil DW.

4.2. Cellular Level

4.2.1. $AgNO_3$ Mechanisms

Overall, an induction of all measured antioxidant enzymes was observed, with the inhibition of CAT being the exception. Similarly, it has been shown that in *C. riparius*, exposure to $AgNO_3$ decreases the CAT activity (Nair et al., 2013). Also, $CuCl_2$ and CuNP have been shown to reduce CAT activity (Gomes et al., 2012), possibly due to direct interaction of Cu with the protein's thiol groups, altering the tertiary structure of

the catalase and inhibiting it (Atli et al., 2006), possibly with a similar mechanism for Ag. On the other hand, CAT has also been reported activated (in other invertebrates) in the presence of $AgNO_3$, e.g., in *Eisenia fetida* (Hayashi et al., 2013a; Gomes et al., 2015), and in *F. candida* when exposed to copper and cadmium (Maria et al., 2014).

The glutathione-related enzymes, GR and GST present different patterns for activation, GR early and GST later induction. It is known that Ag has a great affinity for thiol groups, besides inducing the production of ROS (Behra et al., 2013; Leung et al., 2013; Reidy et al., 2013). Therefore, the presence of Ag can mobilize the GSH levels in the cell (*i.e.*, binding to this substrate) (Hayes et al., 2005; Hellou et al., 2012), so here it seems that an early activation of GR occurred to compensate the unavailable GSH, *i.e.*, oxidized glutathione. The Ag-GSH detoxification is associated with the GST activation, similar to e.g., the detoxification mechanism of Cd (Nakamori et al., 2010), explaining its increase only after 4 days and again after 10 days. Additionally, the initial GR increase followed by a decrease is similar to the response to Cu by *F. candida* (Maria et al., 2014). The GST activity and TG content increase after 4 days may be due to ROS generation, this also related with the LPO levels.

The increase in MT levels must be associated with the Ag chelation. This is in agreement with observations at the gene expression level in *E. fetida* exposed to $AgNO_3$ (Hayashi et al., 2013a) and Cu (Brulle et al., 2007), and *F. candida* exposed to Cd (Nakamori et al., 2010). It is known that Ag can be taken up by Cu transporters and interact with Cu homeostasis, which may contribute to Ag toxic effect (Behra et al., 2013).

Regarding LPO at day 4, this was similar to the response to Cd in *F. candida* (Maria et al., 2014) and Ag in aquatic invertebrates (Géret et al., 2002). This could be the result of the imbalance in the redox in the organisms due to CAT reduced activity, as similarly observed to Cu in *E. albidus* (Gomes et al., 2012). Such reduced CAT activity leads to accumulation of hydroperoxides, which can be removed via the glutathione cycle enzymes. This is reflected in the initial activation of GR, followed by the increase in GST and TG. When the enzymes activity reaches a point of saturation LPO occurs.

4.2.2. AgNP Mechanisms

In contrast to the $AgNO_3$ exposure, CAT activity was significantly increased in the AgNP exposure, except after 6 days, a pattern similarly observed for Cu and Cd in *F.*

candida (Maria et al., 2014). The MT induction occurred after 6 and 10 days, *i.e.*, later when compared to $AgNO_3$. It is unknown if for longer exposure periods this would also be followed by a decrease like in $AgNO_3$.

The increase in the glutathiones (higher GST throughout the exposure length, increased GR after 4 days and the increase in TG after 6-10 days), indicate interactions of AgNP with cytosolic and transmembrane proteins, changing the conformation and impairing the antioxidant defenses (Krug and Wick, 2011; Hayashi et al., 2013b; Saptarshi et al., 2013). Hence, GST levels were continuously high to chelate the radical ligands in thiol groups in glutathione content (Hayes et al., 2005; Hellou et al., 2012; Hayashi et al., 2013a; Leung et al., 2013). The increase in GR was needed to balance the redox potential (GSH recycling), as a result of ROS production from NP interactions (Reidy et al., 2013). Because NPs can also cause DNA damage, leading to synthesis of nuclear GSH, this may explain the increase in the TG content (Markovic et al., 2010; Hellou et al., 2012; García-Giménez et al., 2013).

4.2.3. Comparison of Ag Nano and Ag Salt Mechanisms

As discussed so far it is clear that Ag nano and Ag salt cause dissimilar oxidative stress mechanisms of response (see Figure 3). Differences in response patterns for $AgNO_3$ and AgNP have also been described for e.g., the soil invertebrates *Eisenia fetida* (Hayashi et al., 2013a; Gomes et al., 2015) and *Enchytraeus albidus* (Gomes et al., 2013).

The patterns observed in *F. candida* for GR, TG and MT seem to indicate a delayed effect of AgNP compared to $AgNO_3$ (as shown by some authors (Kittler et al., 2010; Notter et al., 2014)), suggesting an effect caused by the slower release of Ag or a slower uptake. On the other hand, CAT and GST show clearly different patterns, indicating a specific NP effect. As already suggested, AgNPs uptake may be done by different pathways than $AgNO_3$ (Behra et al., 2013; Kaveh et al., 2013; Reidy et al., 2013; Buffet et al., 2014). There seems to be a combined effect of Ag^+ and AgNPs which results in a different time of occurrence of events and consequently a different cascade. This is corroborated by the differences caused in terms of LPO, reflecting previous variations in REDOX enzymes. For instance, following the hypothesis of the Ag^+ release from AgNPs the response of MT, GR and TG could be seen as a delayed response for the AgNP, however this is not the case for CAT, LPO and GST.

5. Conclusions

Oxidative stress was studied for the first time in *F. candida* to AgNPs. Reproduction effect concentrations (EC50) caused dissimilar oxidative stress mechanisms, indicating a combined effect of released Ag^+ (MT and GST) and of AgNPs specifically (CAT, GR, TG, LPO). Ag NM300K were less toxic than $AgNO_3$ in terms of population effects, *i.e.*, survival and reproduction.

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Author Contributions

Luis A. Mendes, Vera L. Maria performed the experiments. Luis A. Mendes, Vera L. Maria, Janeck J. Scott-Fordsmand and Mónica J. B. Amorim conceived and designed the experiment, analysed the data and wrote the paper.

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Chapter three

Multigenerational Exposure of *Folsomia candida* to Silver: Effect of Different Contamination Scenarios (Continuous versus Pulsed and Recovery)

Multigenerational Exposure of *Folsomia candida* to Silver: Effect of Different Contamination Scenarios (Continuous versus Pulsed and Recovery)

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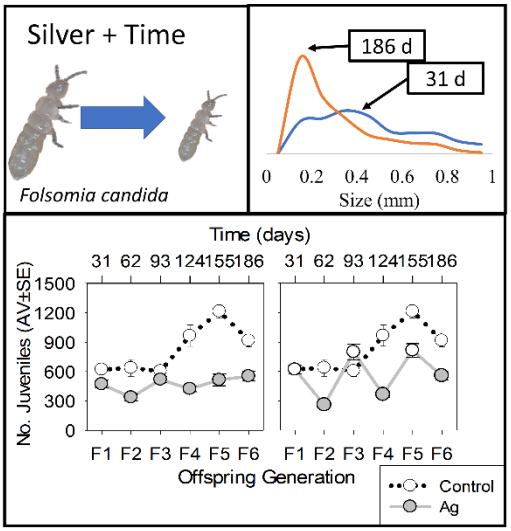


Abstract

Effects of pollutants are mostly assessed using standard testing procedures, which cover a fraction of the animals' life cycle. Although, in nature species are exposed during multiple generations of sub-lethal doses of persistent chemicals. In the present study, we focused on the multigenerational (MG) effects of silver in *Folsomia candida* during 6 generations using the EC50 for reproduction as exposure concentration. We tested 9 different exposure scenarios, going from continuous 6 generations Ag exposure over pulse exposure (i.e. one generation clean, next contaminated, next clean etc.) to gradually increasing the number of exposure generations, with a final transfer to clean media. The biological endpoints assessed included survival, reproduction and size, with

reproduction being the most sensitive. The biological response depended on the specific MG scenario, e.g. the 6 Ag MG caused a decreased number of juveniles from F4, whereas the pulse exposure experienced an increase in reproductive output when in clean soil. It is uncertain whether Ag causes transgenerational effects, but the reproduction levels in both pulse exposures are lower than in continuous control over the 6 generations which could be due to transference of Ag by the maternal generation. Overall, population size distribution seemed to indicate a delay in time for egg laying, with close relationship between adult survival, organisms size and reproduction output. Size monitoring allowed significant added interpretation possibilities and we strongly recommend the addition of this endpoint to the standard guideline. The smaller observed size range can have implications in terms of adaptation potential, carrying associated increased risk.

Keywords: Soil invertebrate; size; area; length, width; transgenerational; multigeneration; long-term;

1. Introduction

Hazard of pollutants in the environment is assessed via standard toxicity guidelines (e.g. from OECD (Organization for Economic Co-operation and Development) or ISO (International Standard Organization), using a battery of key organisms. The common standard procedures target only a fraction of the biota life cycle due to a general choice in restraining the testing time, e.g. using a fraction of reproductive period and assessing survival and reproduction (e.g. 28 days for *Folsomia candida* (OECD, 2009). It is known that in reality, species can be exposed not only over the full life (which current tests do not cover), but also over many generations, both may cause increased impact on population. This is particularly relevant for the terrestrial environment where soil functions as a sink for many pollutants. Persistent toxics may remain attached to soil particles for years, even when some transformations occur (Petruzzelli et al., 2010). Adaptation may occur when organisms are exposed for long to sub-lethal concentrations of contaminants, facilitated by the organisms' phenotypic to genetic plasticity. Such adaptive changes can involve epigenetic mechanisms, i.e., changes in the gene function but not in the DNA sequence (Schlichting and Wund, 2014). These changes can be transferred from one to the next generations (transgenerational), even when the stressor disappears (Klosin and Lehner, 2016). This means that multigenerational exposure can

result in increased resistance or sensitivity to that particular stressor. Obviously, this is not covered by the limited duration of the current standard guidelines, and should receive increased focus.

Few studies focus on the effects of multigenerational (MG) exposure of key organisms, especially when it comes to soil organisms and soil organism exposed to nanomaterials. One example for soil species is the study by Amorim et al (2017) who studied the MG effect of Cadmium (Cd) exposure over 3-years using the collembolan *Folsomia candida*. Amorim et al (2017) showed that for the long-term effects the exposure to lower concentration could cause a higher biological impact than exposure to higher concentrations. The authors suggest this could be due to lower activation of defense mechanisms and a variation in the reproduction size strategy. Another study with the same species (Paumen et al., 2008) showed that effects of MG exposure to phenantrene EC50 (10 generations) caused population extinction at F4. Campiche et al. (2007) showed that F0 exposure to insect growth regulators (methoprene, fenoxycarb, teflubenzuron) caused effects in the 2 subsequent generations after the organisms were transferred to clean media. A study with imidacloprid and thiacloprid (van Gestel et al., 2017) showed differences in terms of MG effects, with the former causing continuous MG effect and the latter causing effects only in F1. Other soil invertebrate MG studies covered oligochaetes, e.g. *Enchytraeus crypticus and E. albidus.* Studies with *Enchytraeus albidus* (Lock and Janssen, 2002) showed that exposure for two generations to Zn, Cd, Cu and Pb did not increase the sensitivity. For *E. crypticus* exposure to Cu caused an increase in sensitivity in the second generation (Menezes-Oliveira et al., 2013). A recent study (Bicho et al., 2017) followed *E. crypticus* exposed to $CuCl_2$ and CuO NM during 4 generations (plus 2 in clean media). Bicho et al (2017) observed two different MG responses, with one Cu form causing an increased sensitivity while the other decreased, this depending also on the test concentration. For this species, epigenetic mechanisms may be involved as methylation analysis showed that 1.4% of the 5-methyl cytosine was methylated (Noordhoek et al., 2017).

These previous studies mostly assumed continuous exposure to the toxic agent, but pollution can occur in pulses or discharges of contaminants, requiring a different design depending on the question and for hazard assessment. For instance, Ag occurs naturally in the environment in low concentrations, mainly in the ionic form of the salt such as silver nitrate ($AgNO_3$) (Purcell and Peters, 1998; Ratte, 1999; Wijnhoven et al., 2009).

Silver is known for its high toxicity in soils (Mendes et al., 2015; Ribeiro et al., 2015) with this being relatively lower after an aging period (Diez-Ortiz et al., 2015). Because the release of Ag containing products in the environment can be delivered to the soil via e.g. sewage sludge treatment (Schlich et al., 2013), a realistic scenario will reflect the input to the system in the form of pulses. In terms of sludge amendment application limits are regulated, e.g. once a year in certain countries or dependent on nitrogen/phosphorous content, but there are other sources like the ones resulting from mining (Candeias et al., 2015) or industrial and urban wastewater discharges (Ho et al., 2012; Johnson et al., 2014). Hence, in the present we studied the MG exposure of Ag to the soil organism *Folsomia candida*, when delivered in a range of 9 different scenarios over 6 exposure generations. The endpoints included survival, reproduction and size (extra to the standard) for all generations.

2. Material and Methods

2.1. Test Organism

Folsomia candida (Collembola) was used. Cultures were maintained in laboratory on a moist substrate of Paris plaster and activated charcoal (8:1 ratio) at 19 ± 1°C, under a photoperiod regime of 16:8 (light:dark). The organisms were fed once a week with dried baker's yeast (*Saccharomyces cerevisae*). Test organisms were of synchronized age (10-12 days).

2.2. Test Chemical, Test Soil and Spiking

Silver nitrate ($AgNO_3$) (99.8% purity, Merck KGaA) was used.

LUFA soil 2.2 natural soil (Speyer) was used. The soil properties can be summarised as follows: pH = 5.5; organic matter = 1.77 %; nitrogen content = 0.17 %; cation exchange capacity = 10.1 meg/100 g; and texture as 7.2 % clay; 8 % silt and 84.8% sand.

Test concentrations were 0 and 145 mg Ag/kg soil dry weight (DW), selected as ca. reproduction EC50 based on a previous study (Mendes et al., 2015), with this being a compromise between a sub-lethal concentration and ensuring a viable number of juveniles to continue the test in the next generation. An aqueous solution of $AgNO_3$ was prepared for spiking. Soil equilibrated for 3 days after spiking, this being repeated at each generation transfer (3 days in advance the soil was spiked). Moisture was adjusted

to 50% of the maximum water holding capacity (maxWHC). Hence, Ag exposure and speciation within each generation was similar.

2.3. Test Procedures

The standard guideline for reproduction test with *Folsomia candida* (ISO, 2014) was used with adaptations. In short, 10 juveniles (10-12 days old) were introduced in the test vessels (Ø 5.5 cm, 250 mL volume) with 30 g wet weight (WW) of soil. Replication consisted of 10 replicates for control and 15 for the Ag spiked soil. Test ran at 20 ± 1ºC, in a 16:8 photoperiod. Food (8mg) and water were replenished weekly. Test duration was extended to 31 days (instead of 28) to allow juveniles to be 10-12 days, matching the start of the first-generation exposure. At test end, the test vessels were flooded with water and gently stirred with a spatula, for organisms to float. The usual procedure for counting was used with a digital picture for analysis using ImageJ Viewer v1.43 image software (Rasband, 1997). To note that besides counting, the size was recorded including body area, length, width and slimness. The juveniles were collected with a spoon, transferred to plaster culture boxes and then selected of similar and larger size (first laid egg clutch). This pool of animals was kept for ca. 2 hours in plaster from which they were randomly transferred to new test vessels for the next generation (10 organisms per replicate again). Exposure was done for 5 generations covering different scenarios as shown in table 1, with all treatments starting at the same time. Although, pulse and continuous Ag exposure may in nature cause different Ag-speciation within the soil core, the current test design alleviated this by having the same exposure regime within each generation. This design allows us to focus on biological changes (e.g. adaption) rather than changes in speciation over time. Hence, speciation will not be discussed.

Table 1: Test design used in the multigenerational exposure of *Folsomia candida* in LUFA 2.2 soil to $AgNO_3$ (145 mg/kg). Ct: control soil; Ag: Ag spiked soil.

Exposure	**Generation**					
Adults	**P0**	**P1**	**P2**	**P3**	**P4**	**P5**
Juveniles	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
6Ct	Ct	Ct	Ct	Ct	Ct	Ct
6Ag	Ag	Ag	Ag	Ag	Ag	Ag
3(Ct-Ag Pulse)	Ct	Ag	Ct	Ag	Ct	Ag
3(Ag-Ct Pulse)	Ag	Ct	Ag	Ct	Ag	Ct
1Ag-1Ct	Ag	Ct	-	-	-	-
2Ag-1Ct	Ag	Ag	Ct	-	-	-
3Ag-1Ct	Ag	Ag	Ag	Ct	-	-
4Ag-1Ct	Ag	Ag	Ag	Ag	Ct	-
5Ag-1Ct	Ag	Ag	Ag	Ag	Ag	Ct

2.4. Image Data Details

Pictures of all replicates were obtained with a fixed position digital camera capturing the surface of the flooded vessels. To ensure comparability across replicates, all pictures were captured at the same distance and angle from the surface where the animals were floating. Further, a size scale was placed at the same level as the floating animals, this could be used to inter-calibration between replicates.

To ensure good detection limits, each picture was captured with a resolution of 55*73 pixels/mm. This prevented size biasing of small animals, since all animals were estimated based on many pixels. No flash or direct light were used, this minimizes the reflection and enhances the identification. The pictures were analyzed using the ImageJ Viewer v1.43 software as follows. Debris was manual edited/removed from the measurement using the software tools. After calibrating the picture with the scale, each photo was adjusted to a 8-bit type and converted into a binary image. Following this, the number of animals were counted and for each animal the measures included area, length, and width.

2.5. Data Analysis

An one-way ANOVA was performed for each treatment, with post-hoc Dunnett's test to test for differences between control and treatments (or between generations) (Sigmaplot, 1997).

Size data was described in terms of average, standard deviation, skewness and kurtosis for all size measurements (area, length, width and slimness). [Note that negative skewness: higher number of large animals (compared to mean) than small; positive skewness: higher number of small animals (compared to mean) than large; positive kurtosis: associated with one clutch of population size; negative kurtosis: associated with more than one average of population size, e.g. 2 clutches.]

Population size distribution was checked for e.g. clutch effects, with the number and size of the bins in histograms calculated following the Freedman-Diaconis rule (Freedman and Diaconis, 1981). The following bin interval for adults and juveniles was used respectively: area 0.2 and 0.02 mm^2, length and width 0.2 and 0.1 mm and slimness 0.5 and 0.2.

3. Results

Multigenerational number of adults and juveniles (figure 1), showed that the validity criteria from the standard test (first generation) were fulfilled, i.e., coefficient of variation for juveniles <30%, number of juveniles > 100 and adult mortality < 20%. Although not standard, the MG results were also within this validity.

There was 10-20% variation on survival between generations. Differences ($p<0.05$) were observed e.g. for all exposures at P5 (see figure for details). Exceptional was a larger decrease in P2 for the 6Ag and the 1Ag-1Ct etc. exposures ($p<0.05$); this particular case did not correspond to a decrease in reproduction or size.

The reproduction output pattern depended on the MG history: 6Ag caused a general lower performance after F3, among other mediated though an increase in control reproduction. The 3(Ct-Ag pulse) caused a clear shift between basal and down response corresponding to Ct and Ag exposure respectively, 3(Ag-Ct pulse), i.e. the opposite order with initial Ag instead of Ct, caused the similar basal-down response, but only from F4 (Ct). The increasing continuous Ag exposure followed by transfer to clean soil

caused a *stimulus* in reproduction when Ag exposed organisms were transferred to clean media, i.e. higher than initial control values.

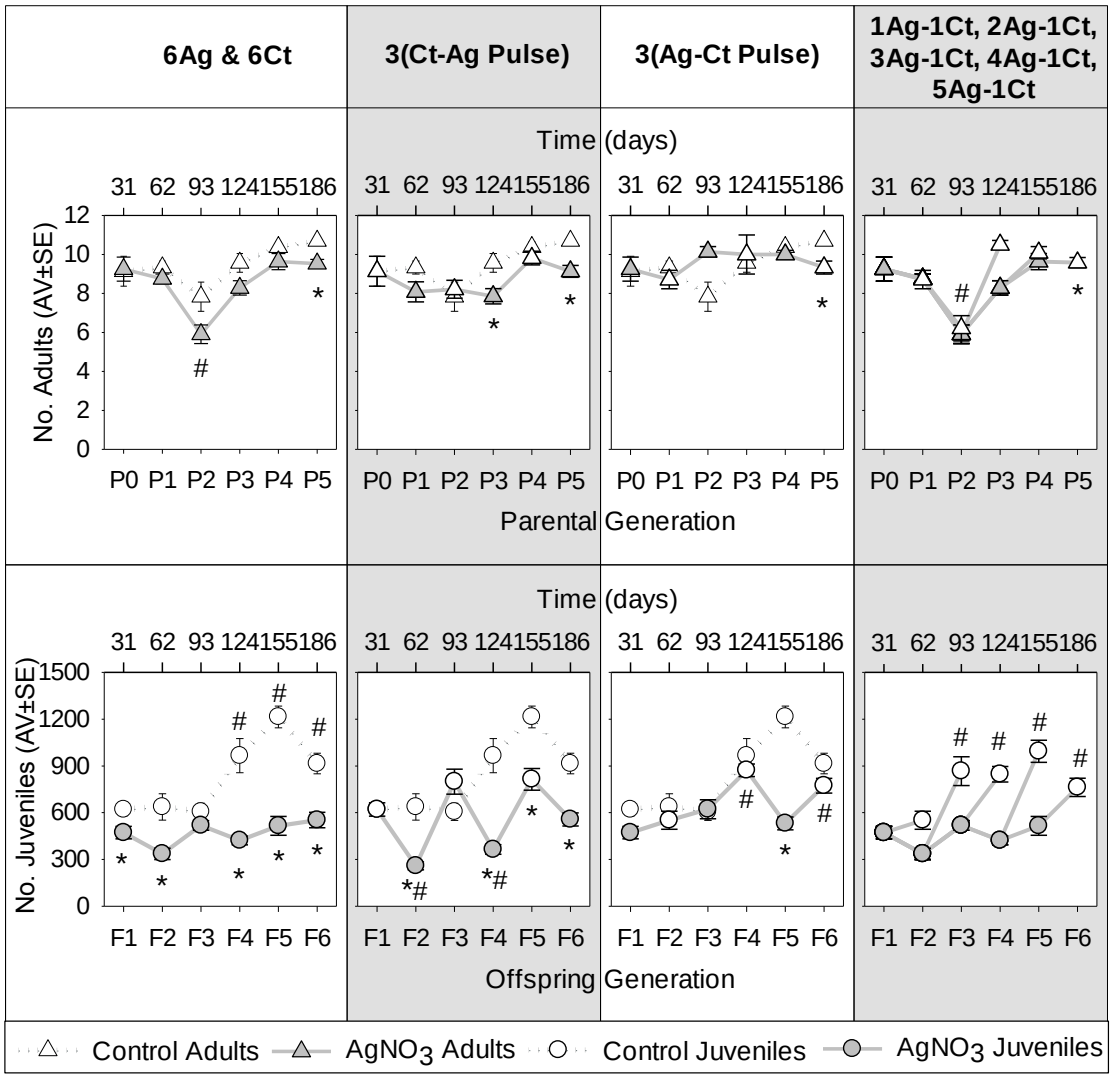
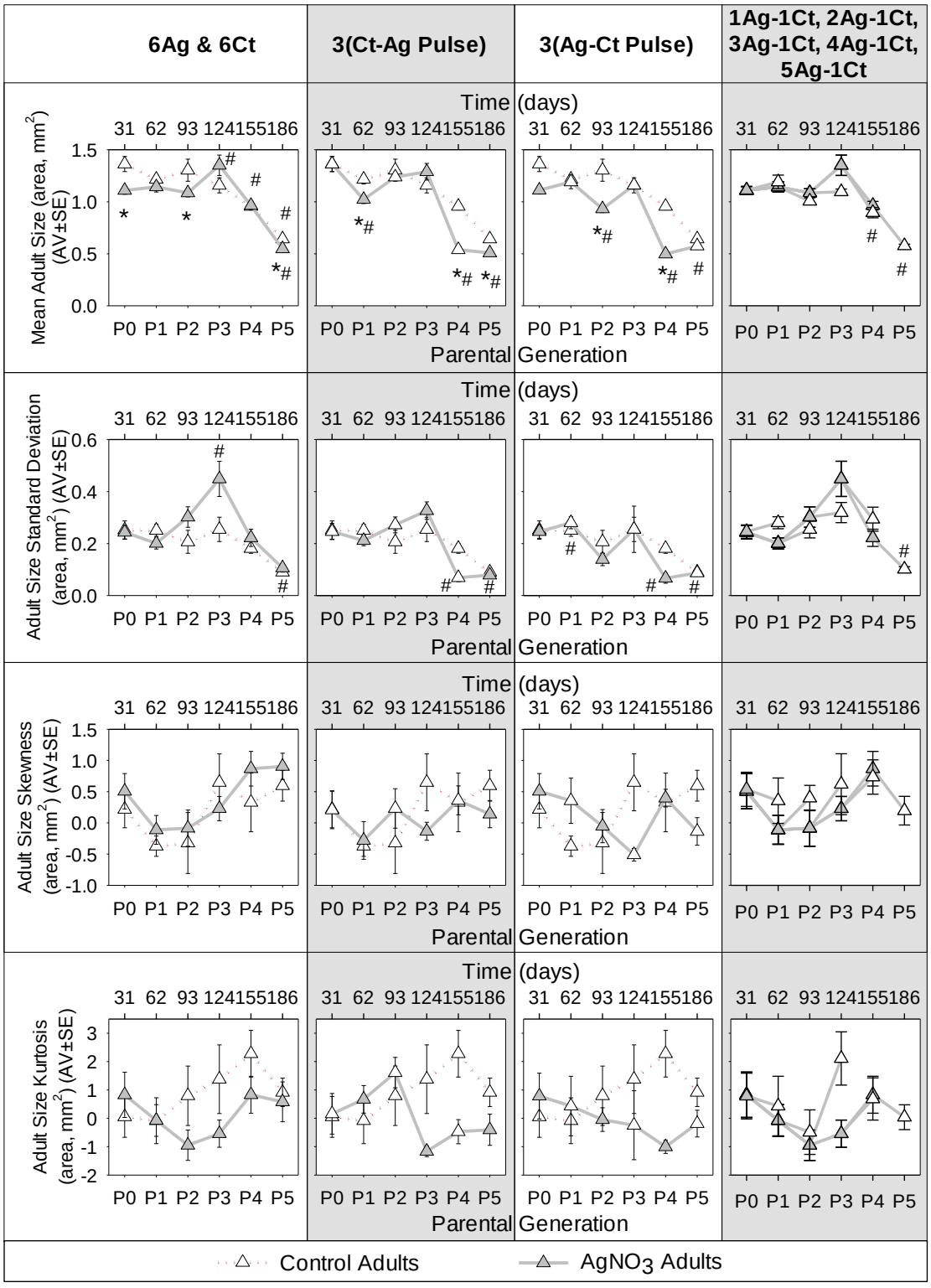


Figure 1: Results of the reproduction test with *Folsomia candida* (endpoints: survival, reproduction) when exposed to $AgNO_3$ (145 mg Ag/kg soil DW) in LUFA 2.2 soil during 6 generations (F1-F6). Average ± standard error (Av ± SE). *: $p<0.05$ between control and Ag. #: $p<0.05$: between F1 and other.

For size (Fig. 2) animals were smaller after P3, in both control and exposure regimes. The standard deviation also decreased, hence the organisms' size is more homogeneous as the generations proceeded. This pattern was similar for all MG tested scenarios. The smaller average size is suggested by a positive and increasing skewness. The positive and increasing kurtosis indicate a distribution of one (peak) population and further supports the smaller variation in size ranges, in this case, towards smaller animals.

While length, width and area show variations with generations, slimness does significantly differ over times (Fig. S1 to S3). This probably indicates that the size variation is proportional, i.e. the organisms did not become “slimmer” or “fatter” for similar length. This is seen both for adults and juveniles (Fig. S3).


6Ag & 6Ct
3(Ct-Ag Pulse)
3(Ag-Ct Pulse)
1Ag-1Ct, 2Ag-1Ct, 3Ag-1Ct, 4Ag-1Ct, 5Ag-1Ct
Time (days)
31 62 93 124155186
Mean Adult Size (area, mm2) (AV±SE)
Adult Size Standard Deviation (area, mm2) (AV±SE)
Adult Size Skewness (area, mm2) (AV±SE)
Adult Size Kurtosis (area, mm2) (AV±SE)
P0 P1 P2 P3 P4 P5
Parental Generation
Control Adults
AgNO3 Adults

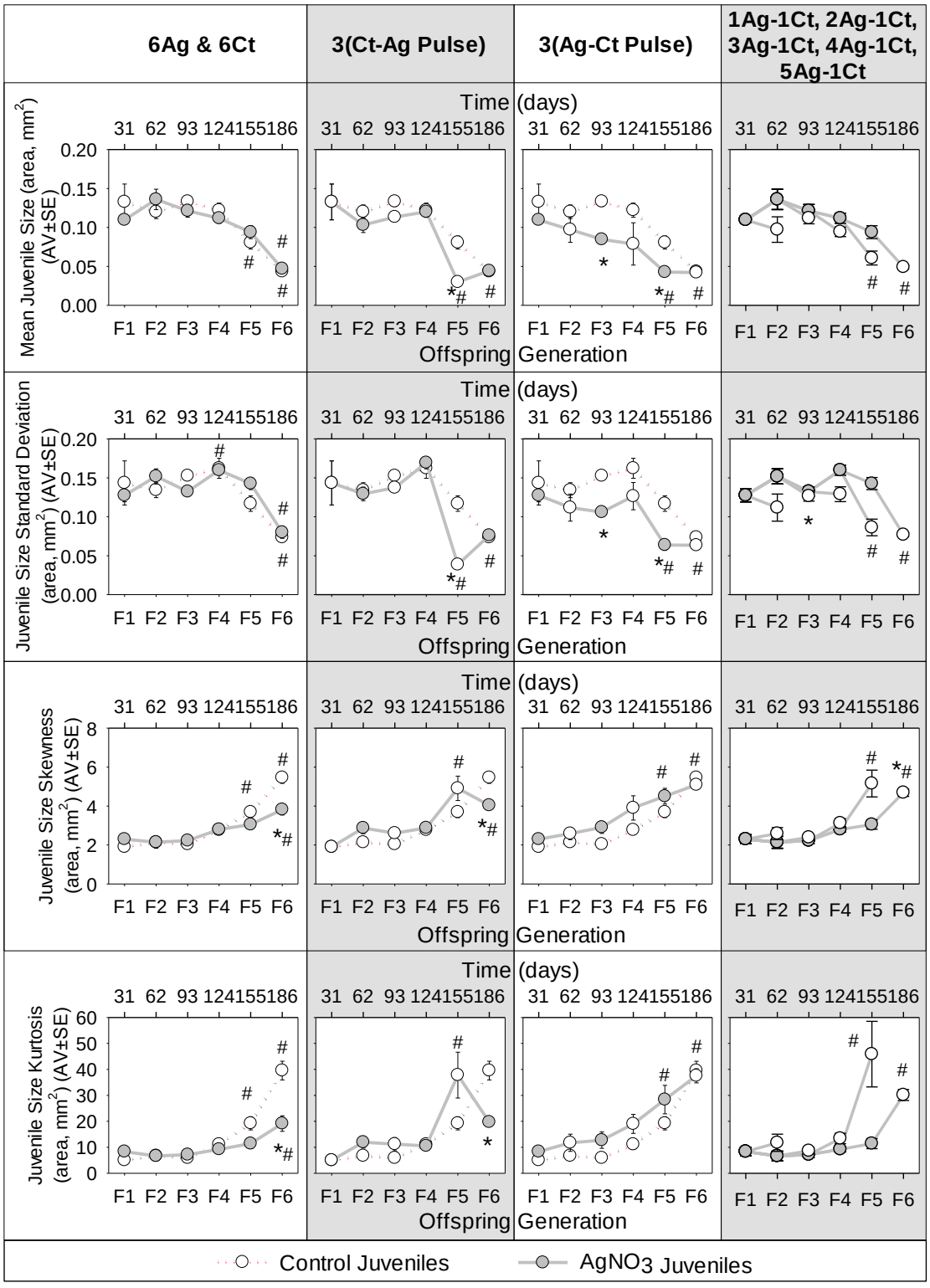


Figure 2: Results of the reproduction test with *Folsomia candida* (endpoints: adults' and juveniles' size (area, in mm^2)) when exposed to $AgNO_3$ (145 mg Ag/kg soil DW) in LUFA 2.2 soil during 6 generations (P0 to P5 parental generation and F1 to F6 offspring generation). Results show mean, standard deviation, skewness and kurtosis. Average ± standard error (Av±SE). *: $p<0.05$ between control and Ag. #: $p<0.05$: between F1 and other.

Screening of organisms' size distribution based on size area (Fig. 3) showed that for adults the size decreased, shifting from an average 1.4mm^2 in F0 to 0.7mm^2 in F6 (for the other size parameters see Fig. S4-S6). The effect was most pronounced in F4, and for both exposed and control. Further, we can see that the size range is smaller, i.e., the sizes are less different. There was a shift in size distribution initially one peak, shifting to two peaks, and then to one peak of smaller animals (i.e. larger animals disappeared). For juveniles, a similar trend occurred, with a shift to smaller juveniles and less of the larger.

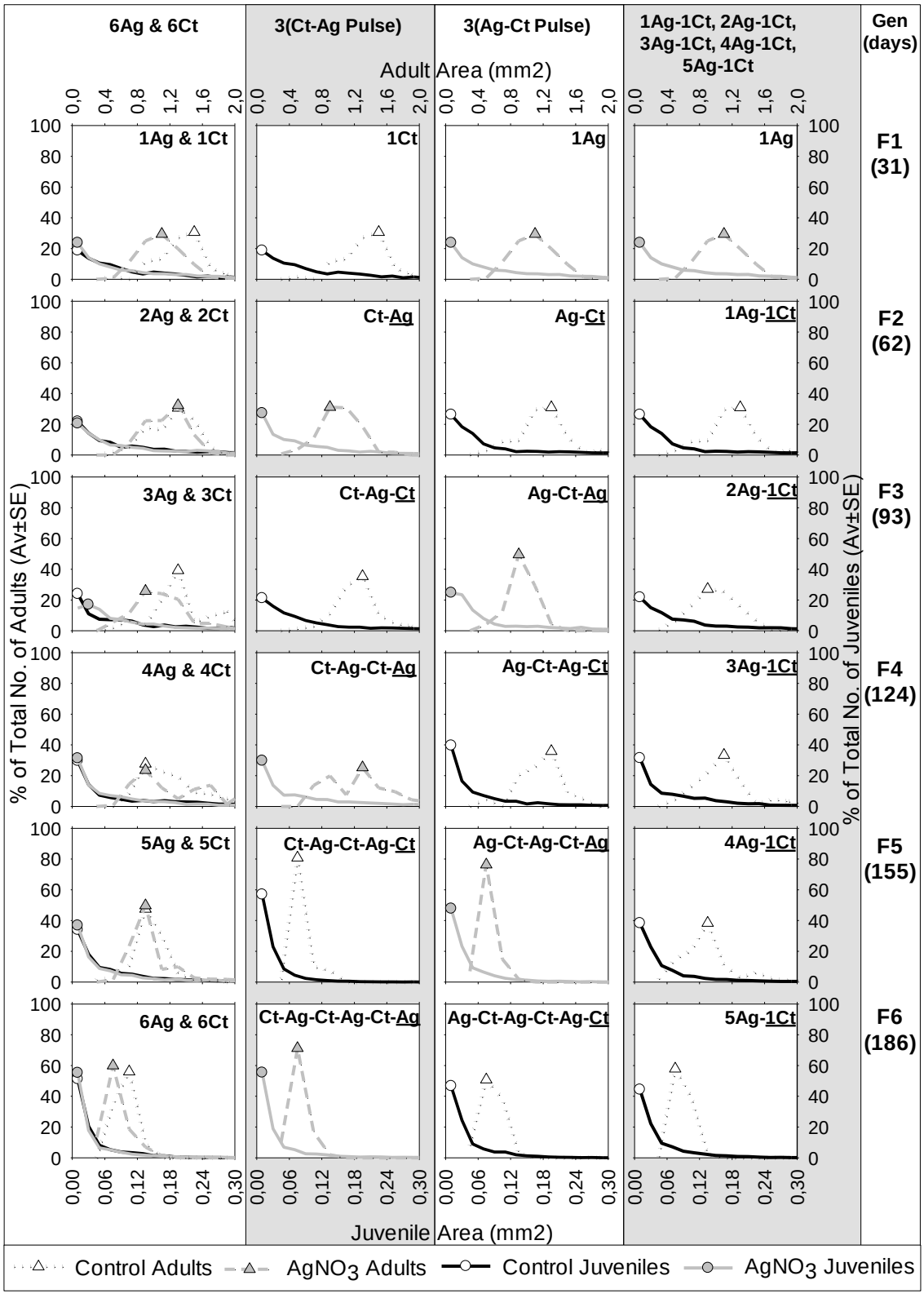


Figure 3: Histogram (endpoints: adults' and juveniles' size (area, in mm^2)) from the reproduction test with *Folsomia candida* when exposed to $AgNO_3$ (145 mg Ag/kg soil DW) in LUFA 2.2 soil during 6 generations (P0 to P5 parental generation and F1 to F6 offspring generation). Results expressed in % of the total number of organisms (adults or juveniles) (AV±SE).

4. Discussion

No significant reduction on adults' survival were observed, as expected, based on the sub-lethal Ag concentration used (Mendes et al., 2015), with the exception of the P2 generation where two treatments had increased mortality. This could be a random event, however since the variation is consistent across generations it is possibly a true effect, for which we have no explanation. The most sensitive endpoint was reproduction, where the responses depended on the MG exposure history. This altogether indicates that reproductive effects observed are not due to direct mortality of adults. On the other hand, the decrease in reproduction occurs after the decrease of size in the adults; it is known that reproductive output may correlate to size (Amorim et al., 2017) (this is discussed ahead in further detail).

There were differences between the various MG scenarios, although not always self-explainable. For instance, the continuous exposure to Ag caused a decreased number of juveniles from F4 onwards. Some differences were more obvious, e.g. when organisms were transferred to clean media there was an increased reproduction pulse, like a recovery.

In a MG exposure to Cd EC50 with *F. candida* (Amorim et al., 2017) an initial increase (F0-F6) followed by a decreased (F7-F12) was observed in number of juveniles, after F12 a low number was maintained for more than 3 years. It is unknown if a similar effect would occur if MG to Ag had been prolonged. Exposure to phenantrene EC50 (Paumen et al., 2008) showed that after four generations there was complete reproductive failure and subsequent extinction. Hence, it seems that the effects of Ag MG were more similar to Cd MG with potential tolerance or adaptation.

For the pulse exposure regimes, there may have been Ag transgenerational effects as the reproduction levels in both pulse exposures were lower than in the continuous control. Previous studies showed that *F. candida* activates the anti-oxidant defenses when exposed to Ag (same EC50) (Mendes et al., 2015). When such defense mechanisms are activated and the stressor is subsequently removed, one of the short-term consequences could be a boost of the compensating effect. Stress mechanisms are known to be turned on – off when in presence – absence of stressor respectively (see e.g. (Amorim et al., 2017b). The gene regulation is the immediate modulator but the protein synthesis requires more time, both to be activated and de-activated, e.g. metallothioneins (Maria

et al., 2014). Hence the phenotypic effects typically last after the stressor is removed, whereas gene expression does not. Here for example there was an increase in reproduction, likely through allocation of extra energy for this function. On the other hand, the size of both adults and juveniles decreased, probably a related tradeoff in energy expenditure.

Results in a MG study with *C. elegans* exposed to Ag (Schultz et al., 2016) suggest a possible increase in sensitivity over generations, although not conclusive given the shifts and variability. The authors suggest the transference of Ag by the maternal generation, which can contribute to a lower reproductive output in sequential generations. If this would be the case, then we should observe a clear but not full decrease of effect in the transfer to clean media. A near full recovery was observed for *F. candida*, which could also be facilitated by the known fast elimination kinetics (Waalewijn-Kool et al., 2014).

Campiche et al. (Campiche et al., 2007b) observed transgenerational effects on reproduction up to F2 after exposure to methoprene and teflubenzuron in F0 (Campiche et al., 2007). Van Gestel et al (van Gestel et al., 2017) showed differences in terms of MG effects with imidacloprid causing continuous effect and thiacloprid causing effects only in F1. MG effects have also been studied using food as the limiting factor (Hafer et al., 2011). A low food availability showed transgenerational (3 generations) effects on the reproduction of Collembolans. This supports the hypothesis of possible epigenetic mechanisms as a mediator of MG effects in Collembolans (Hafer et al., 2011). Although, Noordhoek et al. (Noordhoek et al., 2017) did not find global DNA methylation in *F. candida*, the epigenetic control could occur via other mechanisms.

Interestingly, a study with *F. candida* showed reproductive flexibility depended on the clone (Tully and Ferrière, 2008), i.e., clones could have one of two possible life strategies: 1) HIFLEX: characterized by large mean egg size and reproductive investment, high reproductive investment flexibility, and low adult survival, and 2) LOFLEX: small mean egg size and low reproductive investment, low reproductive investment flexibility, and high adult survival. The divergence could represent adaptations to differing environments, or indicate genetic polymorphism that evolved under physiological tradeoff between reproductive investment flexibility and adult lifespan. It seems that a possible strategy for the organisms in the present experiment is LOFLEX.

In the present study, there was a clear decrease in the size of both the parental and the offspring with MG exposure testing (for both control and exposed). This could be potentially caused by adaptation to the experimental conditions as mentioned in other species and studies, e.g. Schultz et al (Schultz et al., 2016). It should be noted that within the test design here we deliberately did not include long-term Ag-aging, which obviously may be present in soils.

Silver affected size of the organisms in specific generations and depending of the different exposure scenarios (e.g. bigger adults in F4 of 6Ag, smaller juveniles in all generations of 3*(Ag-Ct pulse)). Size effects are shown to occur for other compounds and in a dose related manner, e.g. in *F. candida* to ivermectine (Guimarães et al., 2019), Cd and Al (Crouau and Moïa, 2006), in *C. elegans* to Ag (Schultz et al., 2016) or in *L. rubellus* to As (Anderson et al., 2013). Nevertheless, few published studies report results of size. In the study of Amorim et al. (2017) the observations were that for EC10 MG exposures there was a tendency towards more small animals with time, after which the population collapsed, whereas for EC50 MG exposures, the shift was towards large sized animals without termination. According to Kozlowski and Gawelczyk (Kozłowski and Gawelczyk, 2002) the optimum size is the one that ensures maximum reproduction, and this depends on the adults' survival.

Parameters such as body length, area or slimness of collembolans have been assessed in studies with different aims, such as effects of age (Johnson and Wellington, 1983), soil quality (Marks et al., 2015), Cu (Scott-Fordsmand et al., 1997; Fountain and Hopkin, 2001), Cd (Bur et al., 2012; Amorim et al., 2017) or mixture toxicity (Broerse and van Gestel, 2010; Broerse et al., 2012). For instance, a similar juvenile size distribution as in the first two generations was observed in Cd exposure in different soils (Bur et al., 2010). Our results show that with increasing number of generations, the clutch of juveniles with larger size, tended to disappear. As such, both kurtosis and skewness of the distributions increased. This was also shown to occur in the first 6 generations of a MG study with Cd in transfer to control conditions (Amorim et al., 2017). This suggests that the mechanisms associated with animal size have a variation along time but also that they are chemical specific.

As pointed out above, the lack of a second juvenile peak in size (each peak corresponds to a clutch of juveniles), may indicate that a process of adaptation, delaying the reproductive process, most likely the time for egg-laying, as unhatched eggs were

virtually absent. The first oviposition in *F. candida* occurs after the sixth instar (Snider and Butcher, 2017), with ca. four moulting cycles up to this stage, in an average of 4 days per cycle (Oda et al., 2000). The exposure may affect the first moulting cycles, resulting in a scattered, low percentage, clutch of juveniles, followed later by a second clutch, possibly more adapted.

5. Conclusion

Reproduction and size were sensitive endpoints over MG. There was no systematic increase in mortality with Ag MG. Significant decrease in number of juveniles occurred from F4 (along with decrease in adults' body size) when continuously exposed to Ag. Transfer to clean media allowed recovery of high numbers of juveniles, but with smaller size. It was uncertain whether Ag causes genetic transgenerational effects, as the effects could be due to transference of Ag by the maternal generation. Silver affected size in specific generations and depending on the MG scenarios (e.g. bigger adults in F4 of 6Ag, smaller juveniles in all generations of 3*(Ag-Ct pulse)). Population size distribution seems to indicate a delay in time for egg laying, with close relationship between adult survival, organisms size and reproduction output. Size monitoring allowed significant added interpretation possibilities and we strongly recommend the addition of this endpoint to the standard guideline. The smaller observed size range can have implications in terms of adaptation potential, carrying associated increased risk.

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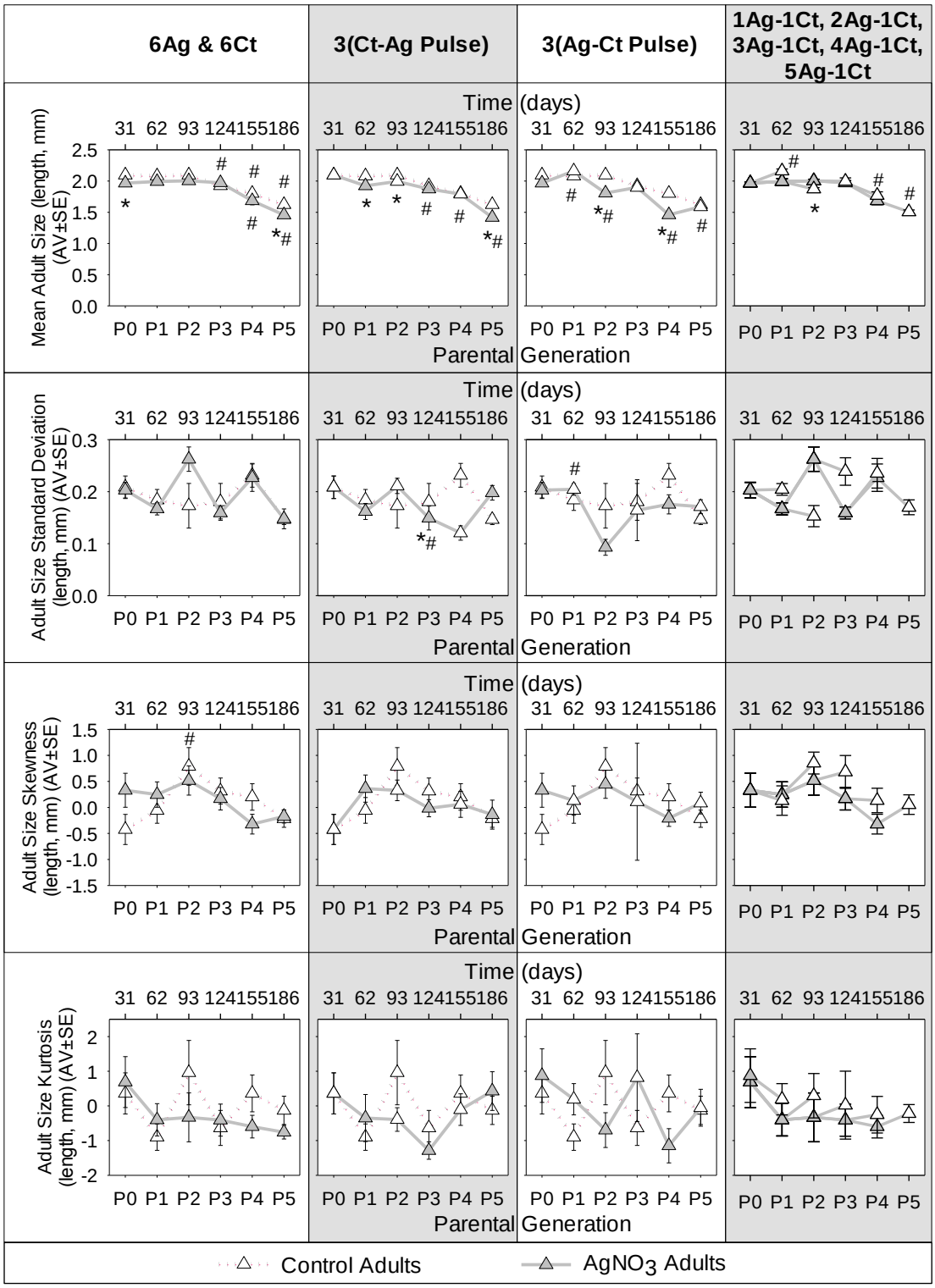
Supplementary Information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Adult Size Standard Deviation (length, mm) (AV±SE)
Adult Size Skewness (length, mm) (AV±SE)
Adult Size Kurtosis (length, mm) (AV±SE)
P0 P1 P2 P3 P4 P5
Parental Generation
Control Adults
AgNO3 Adults

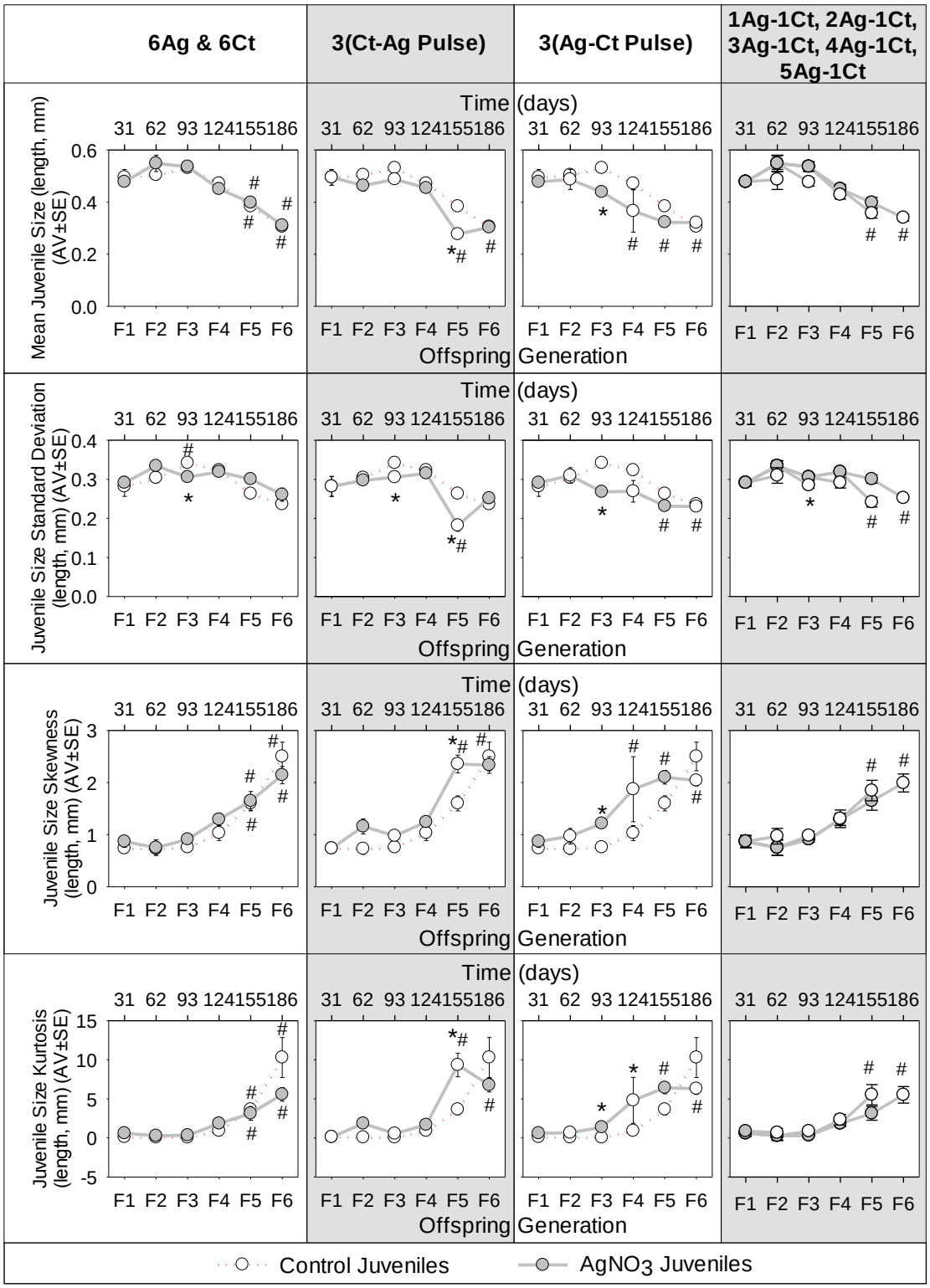
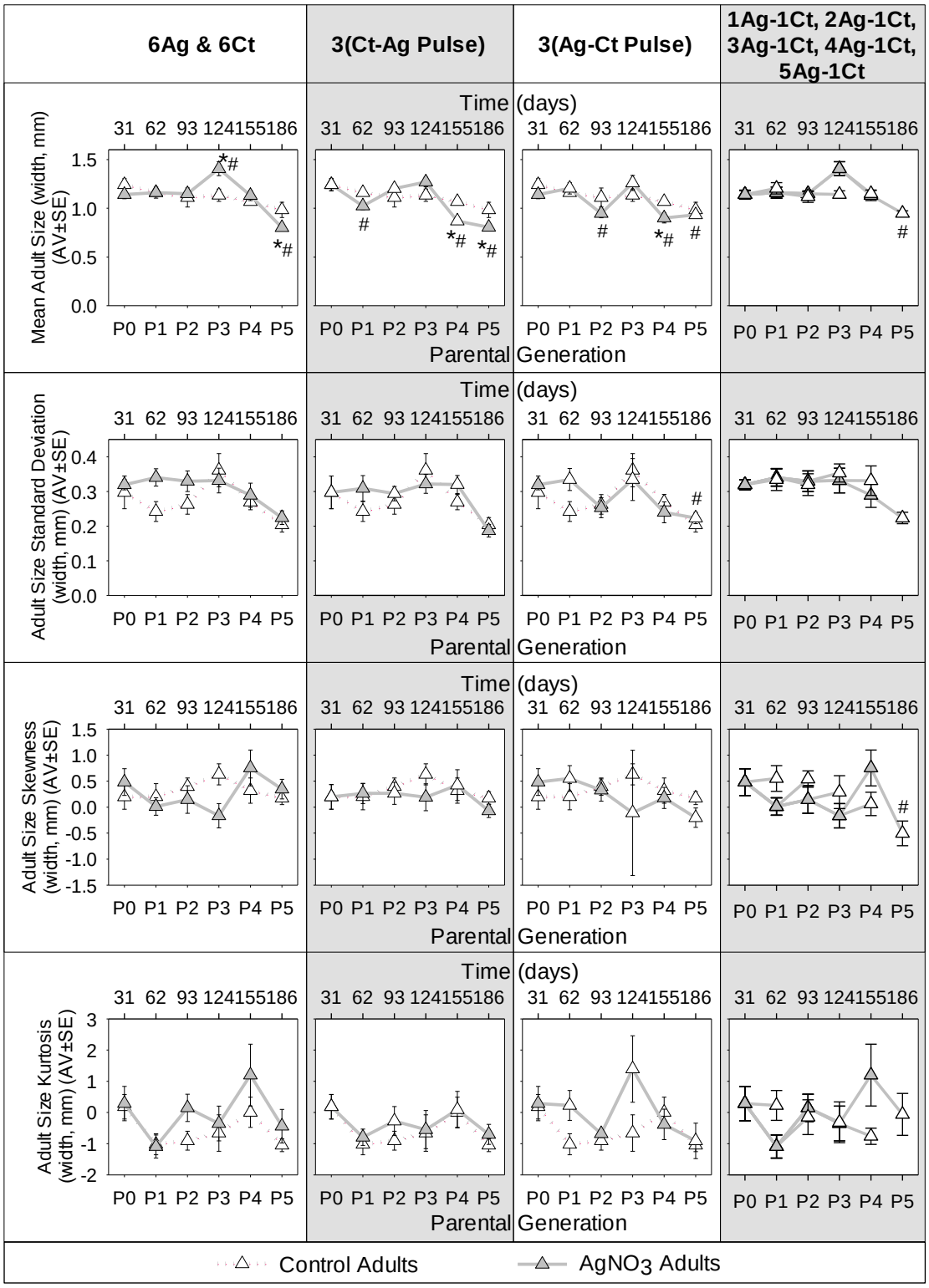


Figure S1: Results of the reproduction test with Folsomia candida (endpoints: adults' and juveniles' size (length, in mm)) when exposed to $AgNO_3$ (145 mg Ag/kg soil DW) in LUFA 2.2 soil during 6 generations (P0 to P5 parental generation and F1 to F6 offspring generation). Results show mean, standard deviation, skewness and kurtosis. Average ± standard error (Av±SE). *: $p<0.05$ between control and Ag. #: $p<0.05$: between F1 and other.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Time (days)
31 62 93 124155186
Mean Adult Size (width, mm) (AV±SE)
Adult Size Standard Deviation (width, mm) (AV±SE)
Adult Size Skewness (width, mm) (AV±SE)
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P0 P1 P2 P3 P4 P5
Parental Generation
Control Adults
AgNO3 Adults

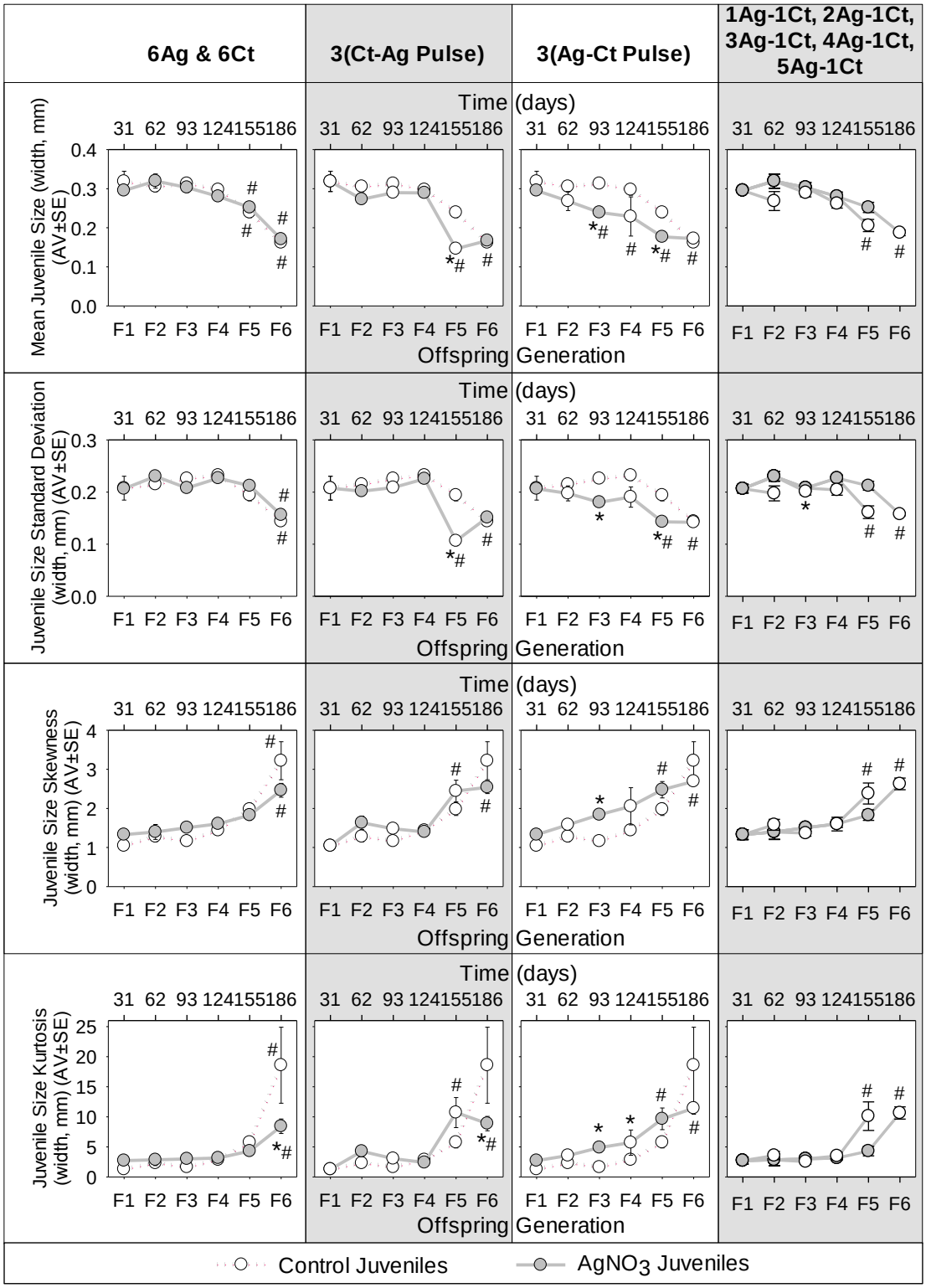
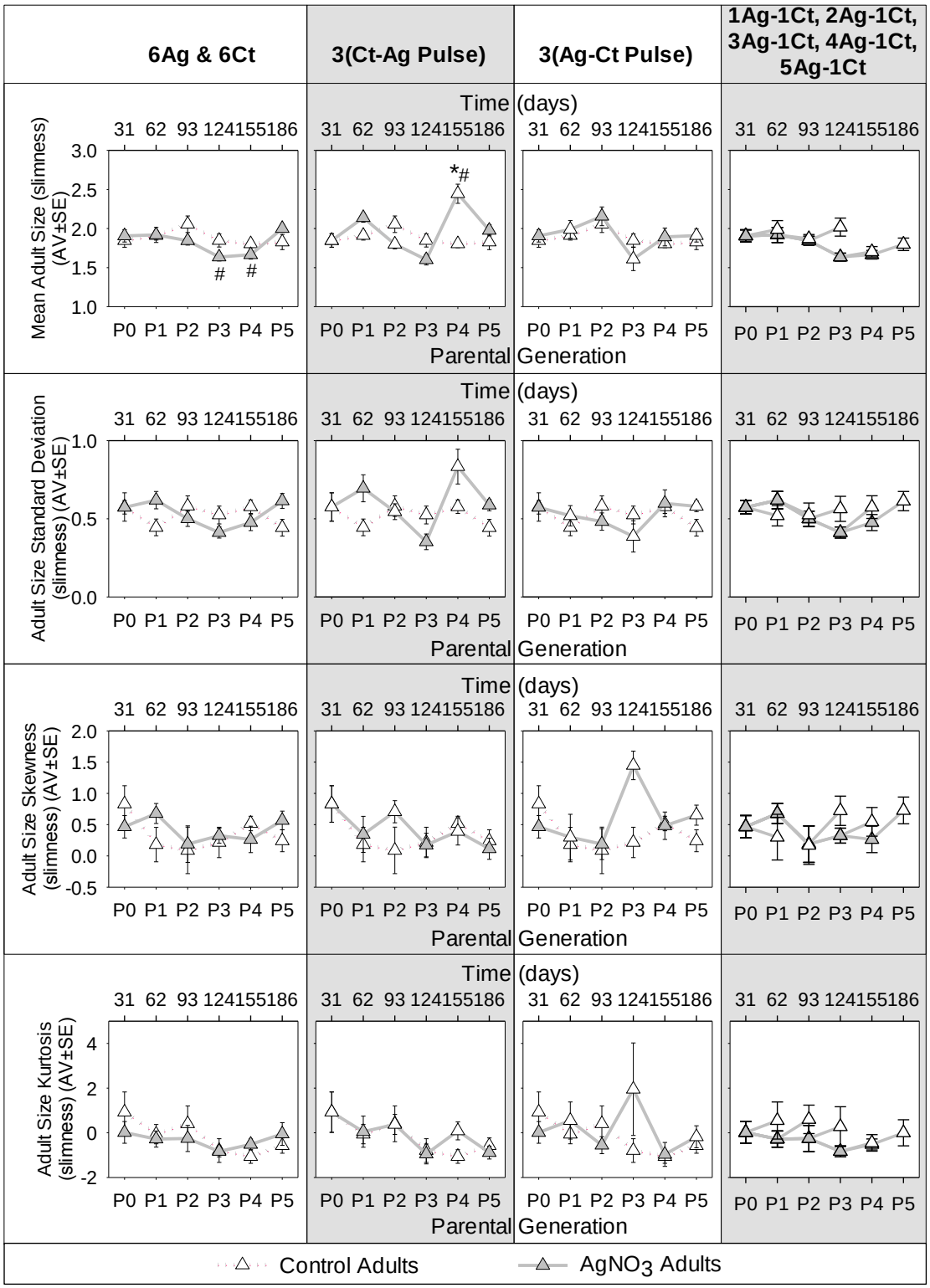


Figure S2: Results of the reproduction test with *Folsomia candida* (endpoints: adults' and juveniles' size (width, in mm)) when exposed to $AgNO_3$ (145 mg Ag/kg soil DW) in LUFA 2.2 soil during 6 generations (P0 to P5 parental generation and F1 to F6 offspring generation). Results show mean, standard deviation, skewness and kurtosis. Average ± standard error (Av±SE). *: $p<0.05$ between control and Ag. #: $p<0.05$: between F1 and other.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3(Ag-Ct Pulse)
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Time (days)
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Mean Adult Size (slimness) (AV±SE)
3.0
2.5
2.0
1.5
1.0
#
#
*#
P0 P1 P2 P3 P4 P5
Parental Generation
Adult Size Standard Deviation (slimness) (AV±SE)
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0.5
0.0
Adult Size Skewness (slimness) (AV±SE)
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1.5
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2
0
-2
Control Adults
AgNO3 Adults

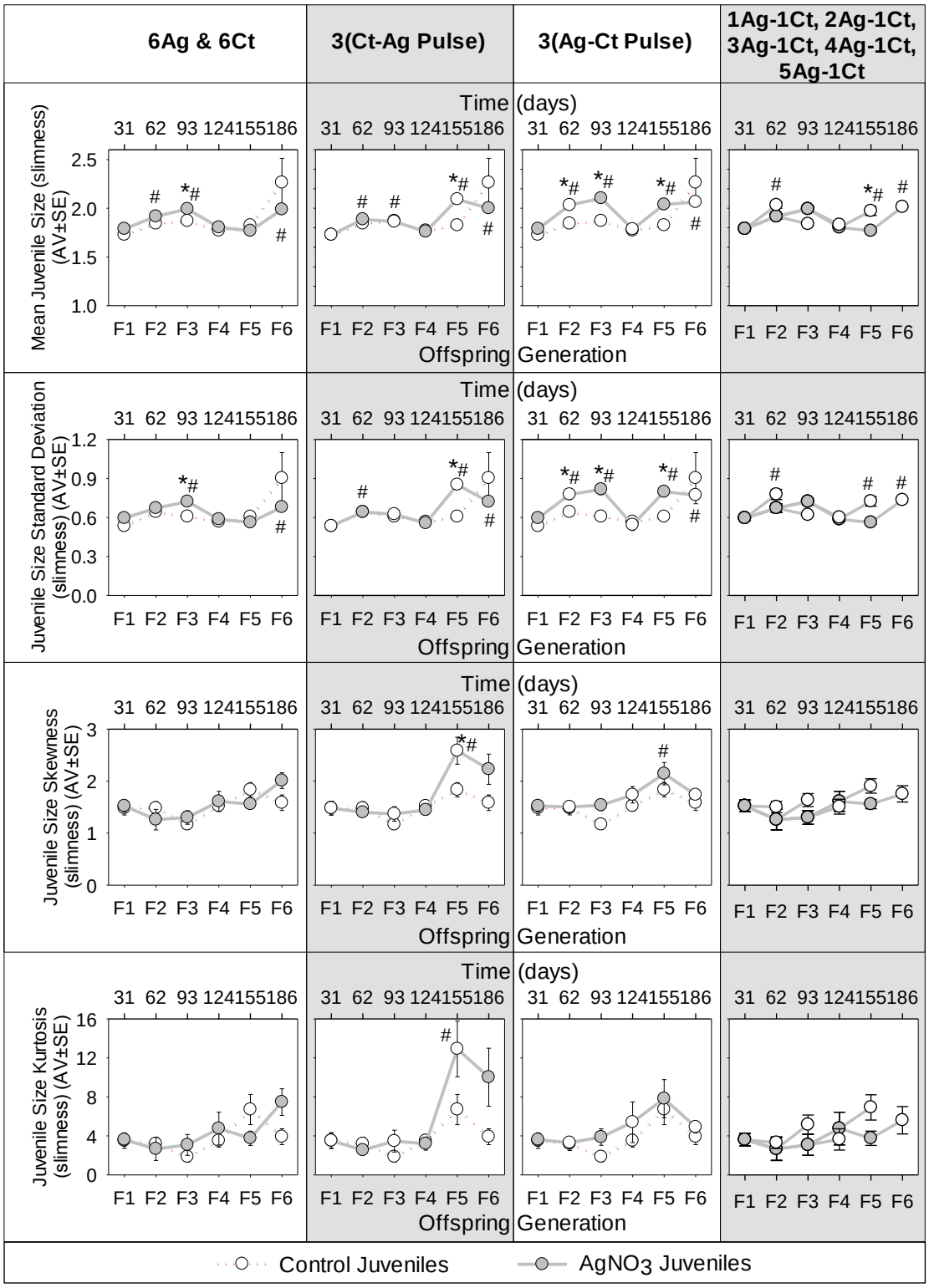

Figure S3: Results of the reproduction test with *Folsomia candida* (endpoints: adults' and juveniles' size (slimness, in length/width ratio)) when exposed to $AgNO_3$ (145 mg Ag/kg soil DW) in LUFA 2.2 soil during 6 generations (P0 to P5 parental generation and F1 to F6 offspring generation). Results show mean, standard deviation, skewness and kurtosis. Average ± standard error (Av±SE). *: $p<0.05$ between control and Ag. #: $p<0.05$: between F1 and other.

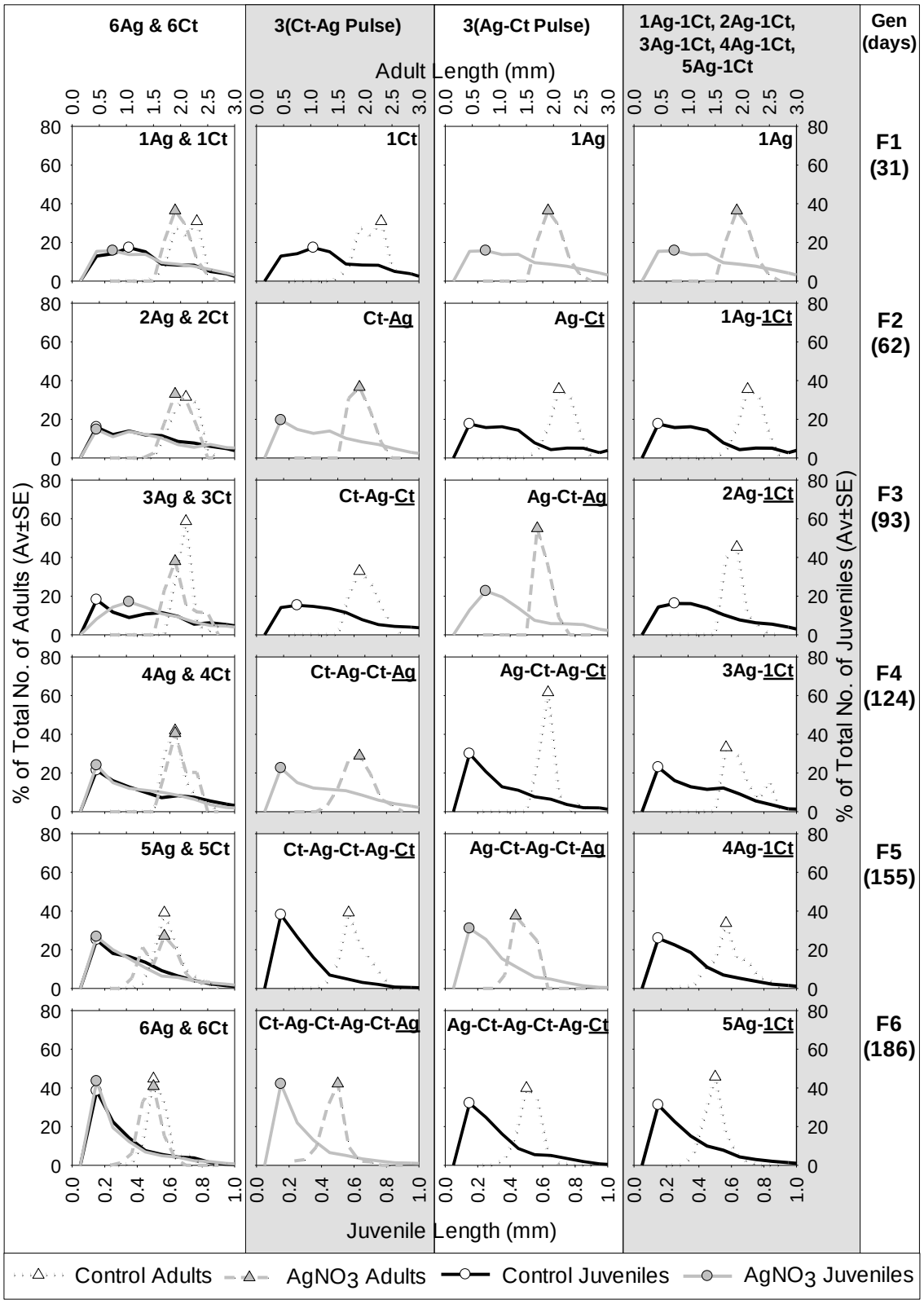

Figure S4: Histogram results (endpoints: adults' and juveniles' size (length, in mm)) for the reproduction test with *Folsomia candida* when exposed to $AgNO_3$ (145 mg Ag/kg soil DW) in LUFA 2.2 soil during 6 generations (P0 to P5 parental generation and F1 to F6 offspring generation) Results expressed in % of the total number of organisms (adults or juveniles) (AV±SE).

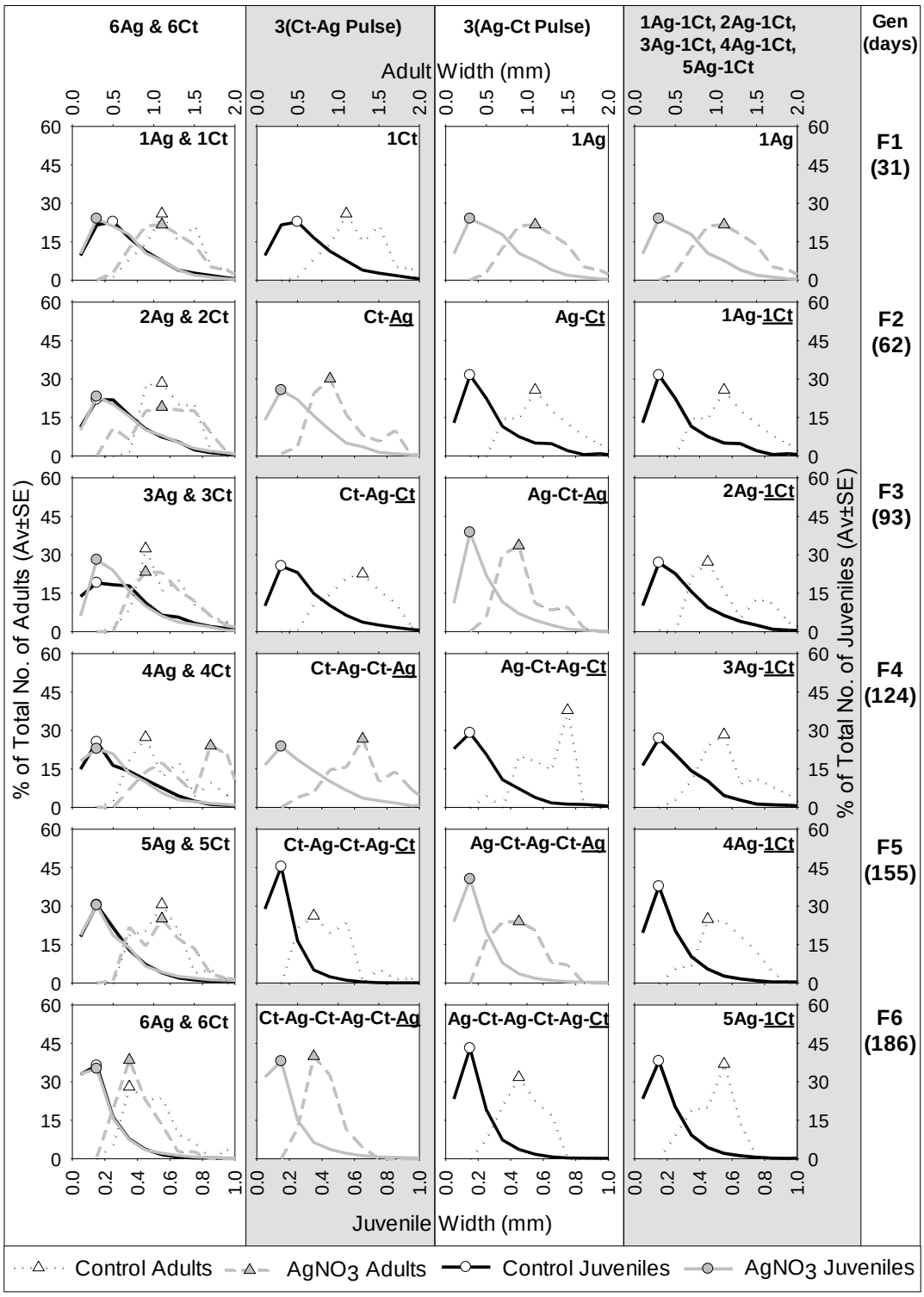

Figure S5: Histogram results (endpoints: adults' and juveniles' size (width, in mm)) from the reproduction test with *Folsomia candida* when exposed to $AgNO_3$ (145 mg Ag/kg soil DW) in LUFA 2.2 soil during 6 generations (P0 to P5 parental generation and F1 to F6 offspring generation) Results expressed in % of the total number of organisms (adults or juveniles) (AV±SE).

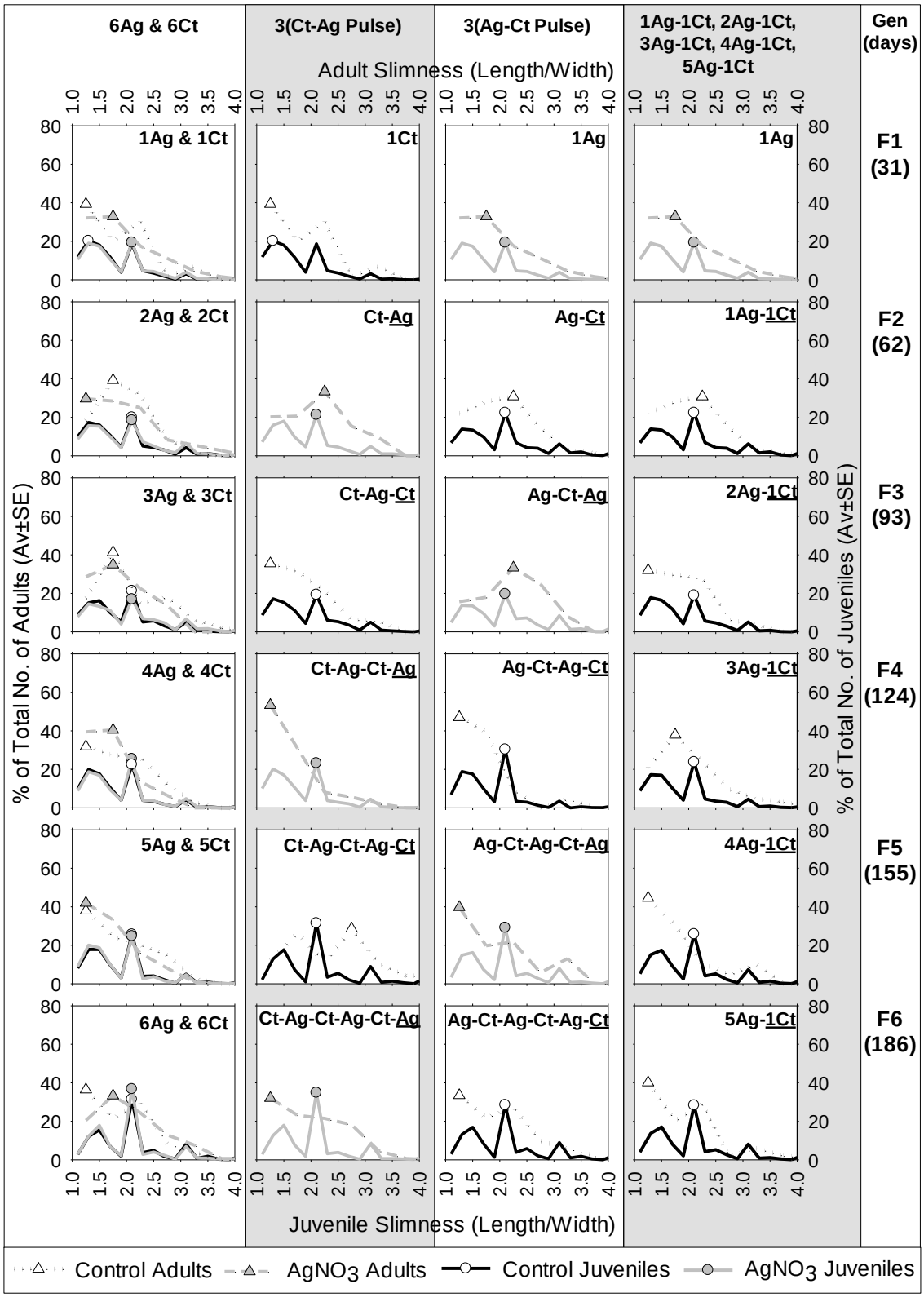

Figure S6: Histogram results (endpoints: adults' and juveniles' size (slimness, in length/width ratio)) from the reproduction test with *Folsomia candida* when exposed to $AgNO_3$ (145 mg Ag/kg soil DW) in LUFA 2.2 soil during 6 generations (P0 to P5 parental generation and F1 to F6 offspring generation) Results expressed in % of the total number of organisms (adults or juveniles) (AV±SE).

Chapter four

Interactions of Soil Species Exposed to CuO NMs are Different from Cu Salt – A Multispecies Test

Interactions of Soil Species Exposed to CuO NMs are Different from Cu Salt – A Multispecies Test

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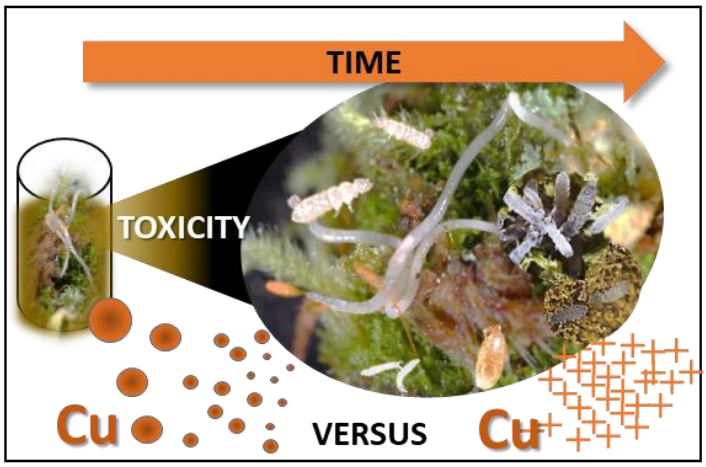

Abstract

Although environmental effects are mostly assessed via standard individual species, the ecological relevance of multispecies testing is well-recognised and highly recommended. Hence, the effect of copper oxide nanomaterials (CuO NM) and $CuCl_2$ were assessed using the validated soil multispecies system (SMS). Besides the individual species (IS) "standard" tests for all, a predation study was done. Toxicity was higher in the SMS than in the IS, and longer exposure showed increased toxicity. The predator (*Hypoaspis aculeifer*) preyed most on smaller species, but the net biomass consumed was similar across species. Internal Cu in *Folsomia candida* reached ca. 140 µg Cu/g dry weight, fluctuating over time, especialy for $CuCl_2$. Copper was mostly bound to soil components for both Cu forms (<0.2% of the total Cu in soil solution, <0.007% on the ionic form, but the soil solution content increased with the total added

concentration). Hazard Concentrations (HC5) showed higher toxicity and more similarity between Cu forms at longer-term exposure. Risk of NMs is relatively limited at present (IS based instead of SMS) with short exposure times (i.e. longer is required) and too few IS tested. The impact of species interactions is highlighted and is of key importance to include in ecosystem hazard prediction.

Keywords: mesocosms; long-term; realistic scenarios; ecosystem function; SSD (Species Sensitive Distribution), HC5 (Hazard Concentration)

1. Introduction

The hazard of nanomaterials (NM) has mostly been assessed using single species studies, e.g. for soil with invertebrates like *Eisenia fetida* (Heckmann et al., 2010; Hayashi et al., 2013a; Gomes et al., 2015a), *Enchytraeus albidus* (Amorim and Scott-Fordsmand, 2012; Gomes et al., 2012a, 2012b, 2013), *Enchytraeus crypticus* (Ribeiro et al., 2015; Bicho et al., 2016, 2017; Santos et al., 2017; Gonçalves et al., 2017) or *Folsomia candida* (Mendes et al., 2015). However, in nature species are virtually never alone and it is well-known that species interact, e.g. via predation, competition, or mutualism. This interaction generates synergistic or antagonystic effects for the community structure and function, which carries key importance for the ecosystem sustainability as a whole (Cameron et al., 2013; Menezes-Oliveira et al., 2013, 2014). Such interactions also affect the hazard and, hence, risk assessment. Although multispecies testing can be more laborious and require more resources than a single species test, it contains key information when it comes to deriving regulatory PNEC (predicted no effect concentration) due to their close resemblance to nature. Previous studies include the soil multispecies systems (SMS) (used to assess the impact of e.g. Cu (Scott-Fordsmand et al., 2008), temperature (Menezes-Oliveira et al., 2013, 2014), biocides (Schnug et al., 2014), pharmaceuticals (Jensen and Scott-Fordsmand, 2012)) and the terrestrial model eco-systems (TME) (Knacker et al., 2004) with a slightly different format but the same purpose (Kandeler et al., 1998; Weyers et al., 2004; Scholz-Starke et al., 2013; Crowther et al., 2015; Pelini et al., 2015). These studies have shown that this higher tier of assessment provides unique knowledge in terms of ecosystem relevancy, as it includes both the interaction between several species and longer time-frames for processes.

In the present study, we used the well-established soil multispecies test system (SMS) to assess the effect of CuO NM and $CuCl_2$. The multispecies consisted of 6 species (1 enchytraeid, 1 mite, 4 collembolans, and microbes). The total test duration was 84 days, with intermediate samples at 28-56-84 days. To support interpretation, the species were also tested individually and in a predatory-prey system, and the Copper was characterised in test media (soil and soil solution) and selected organisms.

2. Material and Methods

2.1. Soil Multispecies Test System (SMS)

A laboratory food-web experiment was prepared based on the SMS design as described in Scott-Fordsmand et al. (2008). The species composition of the SMS (i.e. the actual species selected and the number of organisms added to the soil) represented an agro-ecosystem with six species belonging to different functional and feeding groups, representing species interactions (mutualism, competition, predation, etc.) found in nature. The study was performed using a full factorial design with four ($CuCl_2$) or five (CuO NMs) exposure concentrations, four replicates per concentration, and three exposure durations. The number of organisms was counted individually for all the species in all replicates. In total, 108 SMS units (replicates) were used.

2.2. Test Soil and Spiking

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The main characteristics can be described as follows: pH (0.01 M $CaCl_2$) = 5.5, organic matter = 1.77%, CEC (cation exchange capacity) = 10.1 meq/100 g, WHC (water holding capacity) = 41.8 %, grain size distribution of 7.3 % clay, 13.8 % silt, and 78.9 % sand. Soil was defaunated by drying at 80°C for 48h.

The test concentrations were 0-160-320-640-1280 mg Cu/kg soil (DW) for CuO NMs, and 0-80-160-320 mg Cu/kg soil (DW) for $CuCl_2$, selected based on preliminary tests. This corresponded with previous SMS studies (Menezes-Oliveira et al., 2013, 2014) and individual species studies (Gomes et al., 2015b, 2015c), all targeting the sublethal range for the species.

For CuO NMs, the spiking followed the recommendations for nanomaterials (OECD, 2012; Hund-Rinke et al., 2016) with adaptations: 3 soil batches per concentration were mixed individually with CuO NM as dry powder to obtain the final concentration, this

was done for all exposure concentrations. Each batch corresponded to each of the 3 sampling times used, i.e. 28, 56 and 84 days. Each batch was mixed homogeneously for ca. 30 minutes and then split into 4 replicates. Soil moisture was adjusted to 40% of the maximum water holding capacity (WHC), adding both water and aqueous microbial substrate (see below). For $CuCl_2$, a stock aqueous solution was prepared, serially diluted and spiked into the soil as solution, but otherwise following the description above; moisture was also adjusted to 40% of WHC with aqueous microbial substrate. All soils were equilibrated for 3 days prior test-start.

2.3. Microbial Substrate

The microbial substrate was obtained following Scott-Fordsmand et al. (2008). In short, 1kg of LUFA 2.2 soil was shaken for 3 h with 2l of deionized water then sieved through a 50 μm mesh and diluted 10 times for use. The aqueous substrate was added to the soil after spiking 3 days prior test start.

2.4. Test Materials and Characterization

Copper oxide nanomaterials (CuO NMs) (for details see Table 1) and copper chloride anydrous ($CuCl_2$, >99.9% purity, Acros Organics, Thermo Fisher Scientific, Geel, Belgium) were used.

Table 1: Summary of the main properties of the tested CuO NMs (Source: FP7-SUN, SUstainable Nanothecnologies project).

Properties	**CuO NMs**
Manufacturer	Plasma Chem
CAS number	1317-38-0
Primary size distribution (average)	3-35 (12)
Mode (1st quartile - 3rd quartile) [nm]	10 (9.2-14)
Shape	Semi-spherical
Average crystallite size [nm]	9.3
Crystallite phases (%)	Tenorite 100%
Dispersability in water: D50 [nm];	139.5 ± 4.6;
average agglomeration number (AAN)	346
Dispersability in modified MEM: D50 [nm];	85.2 ± 2.7;
average agglomeration number (AAN)	77
Z-potential in UP water [mV]	+ 28.1 ± 0.6
Isoelectric point [pH]	10.3
Photocatalysis: photon efficiency [unitless]	1.5×10^{-4}
Specific Surface Area [m2 g/1]	47.0 ± 1.7
Pore sizes [nm]	13.5 ± 1.6 (BJH)
	23.0 ± 0.9 (AVG)
Surface chemistry [atomic fraction]	Cu = 0.46 ± 0.05; O = 0.47 ± 0.05
	C = 0.07 ± 0.01
Chemical impurities [mg kg/1]	Na: 505 ± 30; Pb: 36 ± 2 Ag: 13 ± 4

Copper was measured in the soil and in soil-solution following the method details as in (Gomes et al., 2015b) at sampling days 28, 56 and 84. Briefly, the total Cu was measured in soil using Graphite Furnace Atomic Absorption Spectroscopy (AAS-GF, Perkin Elmer 4100, Ueberlingen, Germany), and in soil solution using AAS for the total Cu and using ion-selective electrode (ISE25Cu-9, REF251 reference electrode, Radiometer Analytical, Lyon, France) for the free active form (Gomes et al., 2015b).

For Cu measurements, the samples were acid digested (HNO3 65%) for 3-7 days (see Scott-Fordsmand et al. (2008)). A reference tissue for copper concentration was used (National Institute of Standards and Technology, 1989). The amount of CuO present as nanomaterials in the soil was not determined due to the technical

difficulties/impossibilities, i.e. the particle size was below the detection limit of 15 nm (Navratilova et al., 2015). All soil samples were measured on AAS-flame, while Cu in organisms was measured by AAS-GF.

For the organisms, only *Folsomia candida* was analysed due to issues with sufficient biomass from individual replicates and extractions. At each sampling time (28-56-84 days), 10 *F. candida* were obtained from the top layer of each unit, transferred into a 1.5 ml Eppendorf tube, and snap frozen in liquid nitrogen without depuration. Before the acid digestion step, animal samples were unfrozen and dried at 80°C for 24h to obtain the animal samples' dry weight.

2.5. Test Organisms

Six species were included in the composition of the SMS (see Table 2 for details): four collembolans (*Folsomia candida*, *Proisotoma minuta*, *Hypogastrura assimilis* and *Mesophorura macrochaeta*), one predatory mite (*Hypoaspis aculeifer*) and one enchytraeid (*Enchytraeus crypticus*). All organisms were from well-characterised populations, cultured in our animal laboratory for several years. The initial number of organisms per unit was 30 for each collembolan species, 15 for the mite and 35 for the enchytraeid, consisting of similar sized adult animals all added at test start (day 0), except *H. aculeifer* added at day 7.

Table 2: Overview of the SMS species and characteristics, including adult length size, biomass, reproduction (number of juveniles per adult in a 28 days LUFA 2.2 soil test), function in the system, assumed species interaction type and predicted living layer in the soil.

Sub-class	Oligochaeta	Collembola	Collembola	Collembola	Collembola	Acari
Species	*Enchytraeus crypticus* 	*Folsomia candida* 	*Proisotoma minuta* 	*Hypogastrura assimilis* 	*Mesophorura macrochaeta* 	*Hypoaspis aculeifer* 
Adult length (mm)	7	2.5	1.3	1.2	0.6	1.7
Biomass (mg)	0.25	0.16	0.13	0.12	0.06	0.07 (♀)
Reproduction (juv/ad)	60	120	30	30	15	10
Living layer	Middle	Upper-Middle	Upper	Middle	Surface	Upper-Middle
Function	Decomposer; Fungivore; Grazer; Prey for *H. aculeifer*					Predator
Interaction	Competition; Mutualism; Neutralism; Amensalism					Predation

2.6. SMS Units' Experimental Conditions

The experimental SMS units consisted of polyethylene tubes (33 cm × 9.3 cm ø) having a surface area of 68 cm^2 and sample volume of 2241 cm^3. The tubes, closed on the bottom with a perforated lid, were filled with 1000 g moist soil (800 g dry weight plus deionized water and aqueous microbial substrate). Experiments were performed in temperature controlled rooms (20±1°C) with 12:12h light:dark cycle. The soil water content was maintained by replenishing water loss weekly.

2.7. Sampling and Extraction

At each sampling time (28, 56 and 84 days), 4 SMS units for each concentration and for each material (incl. control) were extracted. The soil of each replicate was divided in 3 equal sized layers, top, middle and bottom, and from each layer ca. 100g (random sub-samples) were used to quantify arthropods, ca. 45g to quantify enchytraeids, 10g to quantify Cu in soil and soil solution. These soil quantities are sufficient to obtain representative samples, based on previous experience (Scott-Fordsmand et al., 2008) The microarthropod sub-samples were extracted over 3 days in a MacFadyen high gradient extractor (Scott-Fordsmand et al., 2000) with temperatures rising from 30 to 60°C. Animals were collected in benzoic acid, transferred to glycerol, identified and

counted. The animals for Cu analyses were sampled alive by flotation of subsample. The enchytraeids were extracted by spreading the soil sample into four 200 ml plastic beakers (ø 7 cm) filled with tap water and ethanol, gently shaken, and then left for 24 h at 5°C for sedimentation. Afterwards, samples were washed and sieved in a 50 μm mesh to extract the enchytraeids for counting.

2.8. Individual Species Tests

Individual species tests were carried out for the collembolans (OECD, 2009), enchytraeid (OECD, 2004; Bicho et al., 2017), and mite (OECD, 2008) following the standard guidelines with adaptations. The CuO NM and $CuCl_2$ exposures used the same concentration range as used in the SMS. The animals (except for the enchytraeid) were not synchronised to resemble the animals selected for the SMS. The tests with collembolans lasted for 28 days and with *H. aculeifer* 21 days. Species were extracted with a MacFadyen high gradient extractor, as previously described.

2.9. *H. aculeifer* Predatory-Prey Test

Hypoaspis aculeifer was allowed to prey on animals from either control (clean) or pre-exposed to Cu. Prey organisms were pre-exposed to the lowest Cu concentration used in the SMS, i.e. 160 mg Cu/kg for CuO NM and 80 mg Cu/kg for $CuCl_2$, to ensure a sub-lethal response. Hence, juveniles of *F. candida*, *P. minuta, H. assimilis* and *M. macrochaeta* were exposed for 7 days as individual species and as a mixture of species, with 80 individuals per vessel at test start. For the mixture of species, 20 animals of each species were used. After pre-exposure, 10 *H. aculeifer* were added to the test vessels, and the predatory-prey test continued for 2 days [based on preliminary tests, 2 days was the optimal time to evaluate predation efficiency]. Endpoint was number of animals and biomass consumed. Samples were extracted for 2 days in a MacFadyen high gradient extractor and collected as previously described. Replicates were extracted into beakers containing plaster and were weighted to calculate biomass consumption.

2.10. Data Analysis

Multivariate: For the SMS experiment, abundance data was analyzed using principal response curves (PRC) based on the redundancy analysis (RDA) (van den Brink and Ter Braak, 1999; van den Brink et al., 2009) using the software package CANOCO version 4.5 (ter Braak and Smilauer, 2002). The significance of the PRC diagrams was tested by

Monte Carlo permutation tests by permuting whole time series in the partial RDA, from which the PRC was obtained. All data were ln transformed prior to analyses.

Univariate: All samples were tested for normality (Shapiro-Wilke test). One way ANOVA was used to assess differences between treatments for data of population (abundance, survival and reproduction), copper concentration, and consumption from the predation test. Post-Hoc Dunnetts', Tukey's or t-test were used for comparisons between control or treatments (Sigmaplot, 1997). For effect concentration (ECx) calculations, based on the measured Cu concentrations, the logistic 2 parameters model used as the best-fit approach using the Toxicity Relationship Analysis Program software (TRAP 1.30) (Erickson, 2012).

Individual species effect concentrations were used to calculate the Hazard Concentrations (at 5% level with a 50% certainty) (HC5). Based on the predicted EC10, the Species Sensitivity Distribution (SSD) and resulting Hazard Concentrations (HC) were calculated using the program EtX version 2.0 (van Vlaardingen et al., 2004), with Anderson-Darling test for normality.

3. Results

3.1. Soil Multispecies Test System (SMS)

In the SMS experiment, the pH was ca. 6.0±0.5 in all treatments and exposure times, with no relation between Cu concentration, time, material or pH.

The overall population (total numbers) abundance curve for controls increased from 0-28-56 days and stabilized from 56-84 days (see Figure S1).

In the Cu-spiked soil, the population growth curve was similar, although with a concentration dependent reduction for total species abundance. There was a significant interaction ($p<0.05$) of all factors, i.e. concentration, exposure time and test material. Toxicity was higher for $CuCl_2$ compared to CuO NM (Fig.S1, Tab.S1). Increased exposure duration in general resulted in a higher toxic impact.

From the redundancy analyses (RDA) (and Principal Response Curves, PRC), it is clear the stressors had a negative impact. For CuO NM exposure, 82% of the total variation could be explained, and within this 49% was explained by time, 51% by exposure concentration, and of this 18% overlapped between both time and concentration. For

$CuCl_2$ exposure, 86% of the total variation could be explained, and within this 49% explained by time, 53% by concentration, and of this 16% overlapped between time and concentration. Monte Carlo permutation test indicated a significant effect [p=0.002, the lowest possible p value for 499 permutations] when testing all concentration exposures versus the control at each exposure time and per material.

All species were negatively affected by Cu (Fig. S1 and S2). Overall, for CuO NMs exposure there were smaller differences between the species (Tab. S1). The order of the species sensitivity was smiliar for $CuCl_2$ and CuO NM, but the relative weight differed (Fig. 1). The species sensitivity was different depending on the exposure time (Fig. S2, Tab. S1). For instance, *H. assimilis* and *M. macrochaeta* abundance decreased the most when exposed to CuO NM, with (i) *M. macrochaeta* being below the initial number, except after 84d, and (ii) *H. assimilis* being nearly extinct after 56 and 84 days. The worm *E. crypticus* was initially (in time) the most affected by $CuCl_2$ (EC10=27mg/kg), but later in time (84 days) this difference was less pronounced. *H. aculeifer* had higher ECx values than for the other species (Tab. S1).

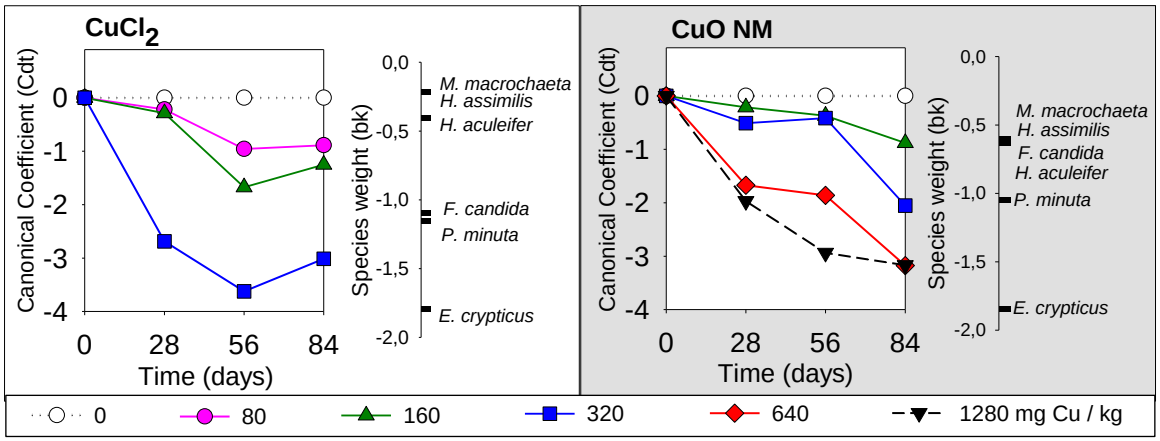

Figure 1: Principal response curves (PRC) with species scores (bk), obtained from the soil multispecies test system (SMS) experiment performed in LUFA 2.2 soil spiked with $CuCl_2$ and CuO NM during three exposure periods (28, 56 and 84 days).

3.2. Individual Species Test

The individual species tests (Fig. S3, Tab.S2) fulfilled the validity criteria as in guidelines: mortality <20%, average number of juveniles >100 for collembolans and >50 for mites, coefficient of variation (CV) <30%. For *P. minuta* and *M. macrochaeta*

CV was >30%. The pH did not vary significantly between test start and test end or between concentrations for either Cu material, being ca. 6.0±0.5.

In regard to mortality and reproductive effects, the individual species showed a decrease with increasing Cu concentrations, with the highest sensitivity for reproduction of *E. crypticus*.

3.3. *H. aculeifer* Predatory-Prey Test

Results of predation test can be observed in Figure S4.

Since the species are of different size and biomass (*M. macrochaeta* is the smallest and *F. candida* the largest), the number of animals consumed does not correspond to the same biomass among species (Fig. S4).

It was observed that the smaller the species, the more individuals were eaten, but the net biomass consumed of each species was similar across species (Fig. S4B/D). There was no difference in consumption between controls and Cu-exposure for *F. candida* and *P. minuta*, but for *M. macrochaeta* and *H. assimilis* more animals were eaten if pre-exposed to CuO NM compared to control (Fig. S4B). In general, there were no differences when comparing consumed biomass in mixed species experiments, similarly smaller species were eaten more than larger. Although, as clearly seen form Fig. S4, the large standard deviation may conceal possible differences.

3.4. Characterization of Cu

The total Cu measured in the soil was ca. 92 ± 15% and 97 ± 30% of the nominal total added concentration for CuO NMs and $CuCl_2$, respectively (Fig. S5 for details). The total in soil solution contained less than 0.211 ± 0.008% of the total measured in soil for both CuO NMs and $CuCl_2$. There was an increase of Cu in soil solution with increase in time, most pronounced for CuO NM highest concentration. The active Cu in soil solution (i.e. Cu^{2+}) was less than 0.007% for both Cu form exposures. Comparing $CuCl_2$ and CuO NM (the overlapping nominal concentrations 160 and 320 mg Cu/ kg dry soil) overtime (Fig. 2), the % of total Cu in the soil-solution was 2-3 fold higher for $CuCl_2$ than for CuO NM spiked soils. The % of free active Cu in the 160 mg Cu/kg concentration increased significantly overtime, with the increase being significantly higher for $CuCl_2$ than for CuO NMs (further details also in Fig.S6).

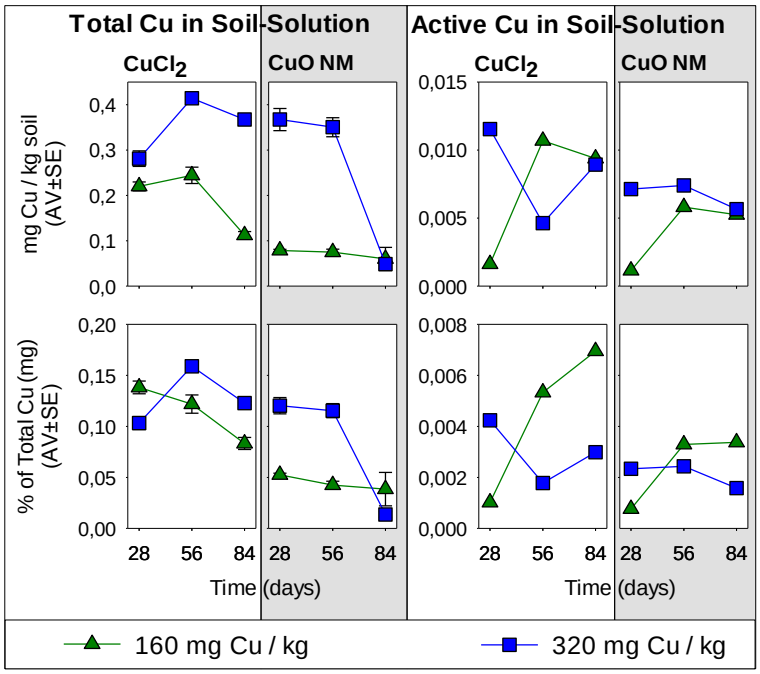

Figure 2: Total Cu soil solution and active Cu in soil solution, obtained from the soil multispecies test system (SMS) experiment performed in LUFA 2.2 soil spiked with $CuCl_2$ and CuO NM during three exposure periods (28, 56 and 84 days). Top rows: total Cu concentration (mg Cu/kg soil DW), bottom rows: relative % of total Cu concentration (mg Cu/kg soil DW). The comparable concentrations 160 and 320 mg Cu/kg soil DW are shown. All results show average ± standard error (n=4).

F. candida internal Cu body concentration was more fluctuating with concentration and time for $CuCl_2$ exposure (Fig. 3).

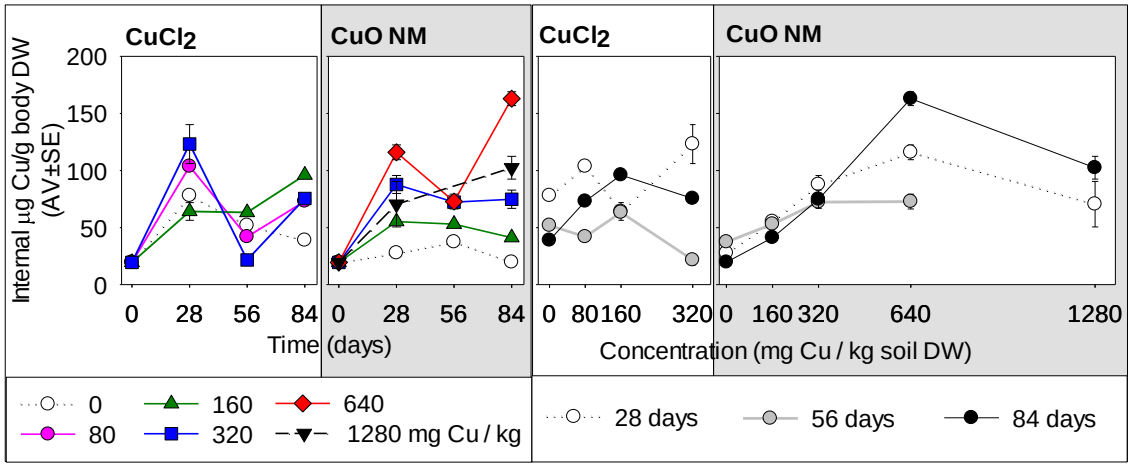

Figure 3: Total internal Cu concentration in *Folsomia candida* from the soil multispecies test system (SMS) exposure performed in LUFA 2.2 soil spiked with $CuCl_2$ and CuO NM in three exposure periods (28, 56 and 84 days). Results are expressed as µg Cu/g body dry weight (average ± standard error, n=4).

For CuO NM exposure, there was an increase up to 150 µg Cu/g organism DW when exposed to 640 mg Cu /kg soil DW. It seems that by day 28, the organisms had reached the accumulation steady state (100 µg Cu/g organism DW), although further uptake was measured at day 84. For concentrations higher than 640 mg Cu/kg DW, there was no further uptake.

3.5. Hazardous Concentrations and Species Sensitivity Distributions (SSD)

The HC5 (hazardous concentration that affects 5% of the species) showed a change in mean values over time with the lowest values for 84 days for both materials (see Fig. 4).

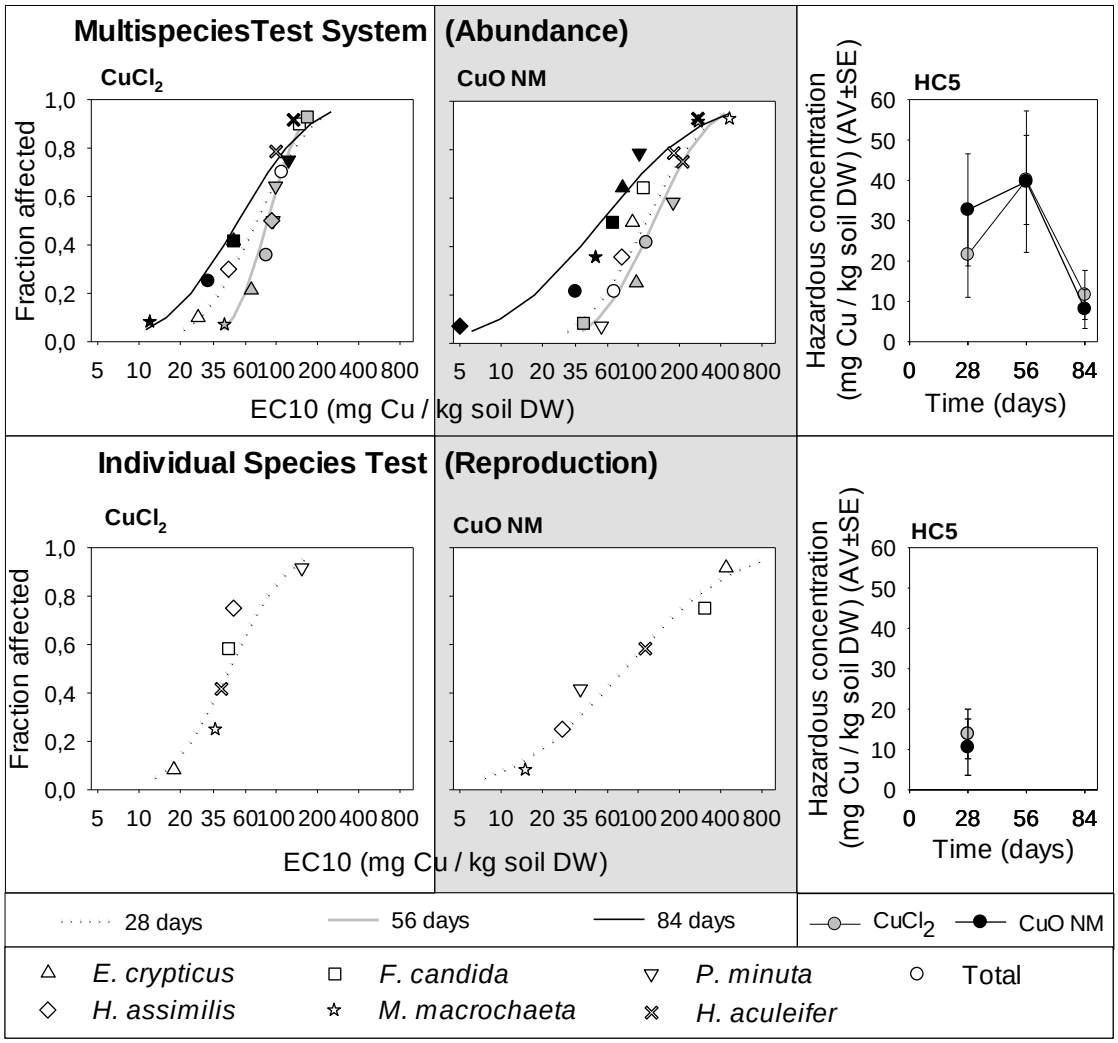

Figure 4: Species sensitivity distributions plot and Hazard Concentrations for 5% (HC5) of the species, as obtained from the soil multispecies test system (SMS) experiment performed in LUFA 2.2 soil spiked with $CuCl_2$ and CuO NM and three exposure periods (28, 56 and 84 days) and the individual species testing (IS).

Based on EC10 values, the HC5 (± SE) for $CuCl_2$ exposure was 21 (±11), 41 (±11) and 10 (±6) mg Cu/kg, for 28, 56 and 84 days, respectively. The comparable values for CuO NM exposure were 31 (±14), 38 (±18) and 6 (±5) mg Cu/kg. For the individual species tests, the HC5 (±SE) for $CuCl_2$ was 20 (±7) and 13 (±6) mg Cu/kg for mortality and reproduction, respectively. For CuO NM, the comparable values were 7 (±6) and 7 (±7) mg Cu/kg.

4. Discussion

One clear observation is that the longer the exposure period (i.e. 84 days) caused higher toxicity. This has been supported by other observations, e.g. full life cycle testing compared to reproduction testing (Bicho et al., 2017). Even longer testing via full life span study with *E. crypticus* (Gonçalves et al., 2017) showed that organisms lived shorter in CuO NM than in $CuCl_2$ when exposed to the respective reproduction EC50s.

For the two Cu-forms (in the SMS) there was no general trend for the EC10 across species, for some species the $CuCl_2$ showed lower ECx values (e.g. *M. macrochaeta*) whereas for other the CuO NM showed lower ECx values (e.g. *F. candida*), both being dependent of time. Hence, $CuCl_2$ results does not mirror CuO NM effect levels. For both exposures the species ranked differently in sensitivity between the multispecies and the single species system (28 days comparison). Hence, in the multispecies system interactions between species were present. The species *E. crypticus* was the most sensitive in all the exposures, which has been observed in previous studies involving enchytraeids and collembolans (Scott-Fordsmand et al., 2008). In particular, for the CuO NM exposure the EC50s for most species were lower in the SMS compared to the EC50s in the IS, which confirms the influence of species interactions for toxicity. While this may seem natural and expected, it highlights the importance of an SMS approach for an ecosystem tier of understanding as opposed to the IS testing. In addition, as shown in a previous SMS study, the changes in community structure were unpredictable for interaction between climate change and Cu (Menezes-Oliveira et al., 2013) because the different species in an ecosystem have varying limits depending on the stressor, which greatly changes the interactions thereafter. Further, the SMS design has a time metric so we can compare 28, 56 and 84 days. Whereas the total species abundance tells us that there was a small increase of toxicity from day 28 to 84, the individual species abundance (in the SMS) showed that these can reflect a larger impact on particular

species (*E. crypticus, H. assimilis*) and smaller in others (*F. candida*), while also depending on the Cu form. For instance, while *F. candida* seems to be as sensitive to Cu as *P. minuta*, there are clear differences in abundance and contribution for the whole population (Fig.S7 and S8). This is probably a consequence of the known higher reproduction rate of *F. candida* compared to other collembolan species. The high reproduction rate of *F. candida* results in a higher increase in abundance despite being predated by *H. aculeifer*, as also observed by (Cortet et al., 2003; Scott-Fordsmand et al., 2008; Menezes-Oliveira et al., 2013, 2014). The predation test showed that all collembolan species are prey for *H. aculeifer*, but the smaller ones (*H. assimilis* and *M. macrochaeta*) are eaten in higher numbers. There is evidence that, besides their smaller size, *M. macrochaeta* have a lower mobility (Ponge et al., 2006), becoming more prone to be preyed on. This was even more pronounced when the organisms were exposed to Cu. Needless to say, this will carry implications for the final outcome in the ecosystem.

Together with previous studies, our results point to predation being one of the factors explaining the decrease in abundance for *H. assimilis* and *M. macrochaeta* over the course of the experiment and the general decrease in the estimated ECx values in the SMS. Previous SMS studies with Cu have also shown a depletion of *H. assimilis* when exposed to Cu (Menezes-Oliveira et al., 2013, 2014). Hence, predation seems an important factor when estimating effects on ecosystems.

The species scoring shows that *M. macrochaeta* is the least affected by Cu, which corresponds with reports that *M. macrochaeta* are tolerant to Cu-contaminated soil and are able to repopulate a defaunated site (Filser et al., 2000). The increase in abundance after 56 and 84 days in the $CuCl_2$ and CuO NM exposure is accompanied by the decrease in abundance for *P. minuta* and *H. assimilis*. The species' decrease in abundance, likely due to predation as suggested before (Scott-Fordsmand et al., 2008), may allow *M. macrochaeta* to repopulate the contaminated site. On the other hand, the lower abundance of *M. macrocheata* throughout the experiment can be explained by the high predation by *H. aculeifer* on this species. Hence, for both copper form exposures there is apparently less impact by exposure compared to predation, as presented in the species score graph.

Overall, the hazard concentration (HC5) was lowest after 84 days for both materials, indicating generally increased toxicity over time. However, over time (28, 56 and 84

days) the sensitivity of the individual species, involved in the Multispecies testing, changed, which makes it problematic to have firm statements on the general sensitivity of individual species.

The measured concentrations of total copper in soil or soil-solution indicate that virtually all the copper attaches to the soil matrix. Similar observations have been reported by Gomes et al. (2015b). Nevertheless, if one zooms in on the results of the comparable Cu concentrations between $CuCl_2$ and CuO NM, for e.g. 160 mg Cu/kg DW, the total Cu concentration in the soil solution and as active ions is 2 fold higher for $CuCl_2$ than for CuO NM exposure. This could explain the lower effects of CuO NM, assuming toxicity is due to Cu^{2+}. However, it seems that toxicity of CuO NM is also caused by the nanoparticulate Cu itself, given the increased toxicity in the longer-term exposure, as no correlation was observed between the species abundance and the measured Cu concentration in soil solution. It has been shown for CuNPs that in the presence of organic matter (OM) the NPs themselves causes toxicity; this is explained by the large reduction of the concentration of Cu ions (released from CuNPs and bound to OM) and the increased stability of Cu as NPs (Xiao et al., 2018).

Wang *et al.* (2016) have shown that the antibacterial effect of CuO based NM is a result of both the released Cu^{2+} and the non-dissolved CuO NM. Several studies with nanometalic particles and respective salts have pointed to an increased effect with increased time for NM (van der Ploeg et al., 2014; Mendes et al., 2015; Ribeiro et al., 2015; Bicho et al., 2016, 2017).

Besides a possible internal transformation of the NMs, the high CEC of LUFA 2.2 soil promotes the attachment by positively charged ions, such as Cu^{2+} and Cu^{+} or the positively charged CuO NMs (zeta-potential + 28.1 mV), in exchange for existing cations, such as H^{+} or Na^{+}, as previously confirmed (Julich and Gäth, 2014; McShane et al., 2014). At the same time, other transformations (beside dissolution), e.g. aggregation and agglomeration (Lowry et al., 2012; Cornelis et al., 2014), are likely to take place in the soil and its aqueous biofilm. It has not been possible to quantify how much of the attached Cu in the soil matrix or in animals is in nano or dissolved form due to limitations related to size particle (<15 nm) for Single Particle-ICPMS (Navratilova et al., 2015).

Nanoparticles are expected to transform once inside the organisms, either because of increased dissolution in low pH media (Jiang et al., 2014) or the formation of corona when entering in contact with proteins (Hayashi et al., 2013b, 2013a), facilitating the crossing of cell membranes and possible Trojan horse-like mechanisms (Park et al., 2010; Xu et al., 2015). The internal body Cu concentration in *F. candida* showed an increase in uptake of CuO NM up to 640 mg /kg soil (100-140 µg Cu/g organism DW for 28-84 days, respectively), and no further increase with exposure to higher concentration (1280 mg Cu/kg). This suggests that a threshold level was reached and that the mechanisms that regulate Cu homeostasis (Cu is an essential element) and detoxification were activated (Ardestani et al., 2013), namely Cu transporters (Puig and Thiele, 2002; Bertinato and L'Abbé, 2004). In the exposure to $CuCl_2$, the accumulation pattern was less stable (28d), but it also indicated that organisms reached a plateau at ca. 140 mg Cu/kg soil (56-84d). This could be because the concentration range was below the threshold for the accumulation peak peak as suggested by other authors (Ardestani et al., 2013; Ardestani and van Gestel, 2014). In these accumulation studies, the internal Cu concentrations in *F. candida* did not exceed ca. 300 ug Cu g^{-1} dry body weight with a slight increase over time and a stabilization after 15 days, suggesting Cu is regulated by collembolans (Ardestani et al., 2013). It has also been suggested that Cu regulation in *F. candida* involves the limitation of uptake rather than active excretion (Ardestani and Gestel, 2012; Ardestani and van Gestel, 2014). The lack of steady patterns may also reflect the differences in organisms' age (and molting events) in the samples of an SMS design.

Adding this to the observation that Cu accumulation increased with exposure time for CuO NM (highest measured after 84 days), this supports the potentially higher toxic effect of CuO NM with time.

In summary, for the two Cu forms the toxicity decreased at intermediate timepoints and then increased after 84 days. For the two Cu-forms there was no general trend for the EC10 across species, for some species the $CuCl_2$ showed higher toxicity whereas this was the case of CuO NM for other species. Hence, $CuCl_2$ results do not mirror CuO NM effect levels. For both exposure the species ranked differently in sensitivity between the multispecies and the individual species system. This indicate that in the SMS test the overall toxicity is not just a sum of the individual species toxicity, but rather due to a

community interaction. Although this point is well-known in nature, it is difficult to capture in laboratory experiments. Soil fate measurements confirmed previous observations that Cu is mostly attached to soil particles, with minute fractions in soil solution and even less as active Cu. The biota Cu uptake tended to increase with exposure time for CuO NM, hence, a potentially higher toxic effect is expected for longer-term exposure of CuO NMs. These results carry major consequences in terms of risk assessment and highlight the need to revise and adapt the requirements for safe assessment of NMs.

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Supporting Information

Figures (S1 to S8) and tables (S1 and S2) are provided in supporting information.

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Supplementary Information

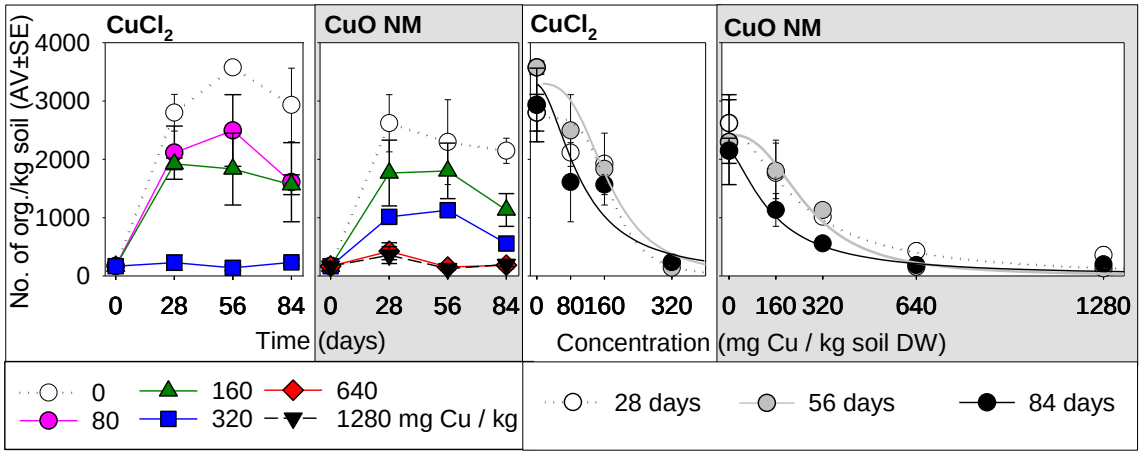

Figure S1: Effects of $CuCl_2$ and CuO NM in terms of total abundance obtained from a soil multispecies test system (SMS) experiment performed in LUFA 2.2 soil spiked with CuO NM and $CuCl_2$ during three exposure periods (28, 56 and 84 days). Results are presented as total number of organisms per kg (average ± standard error, n=4). Lines represent the curve fit to model (logistic model, 2 parameters).

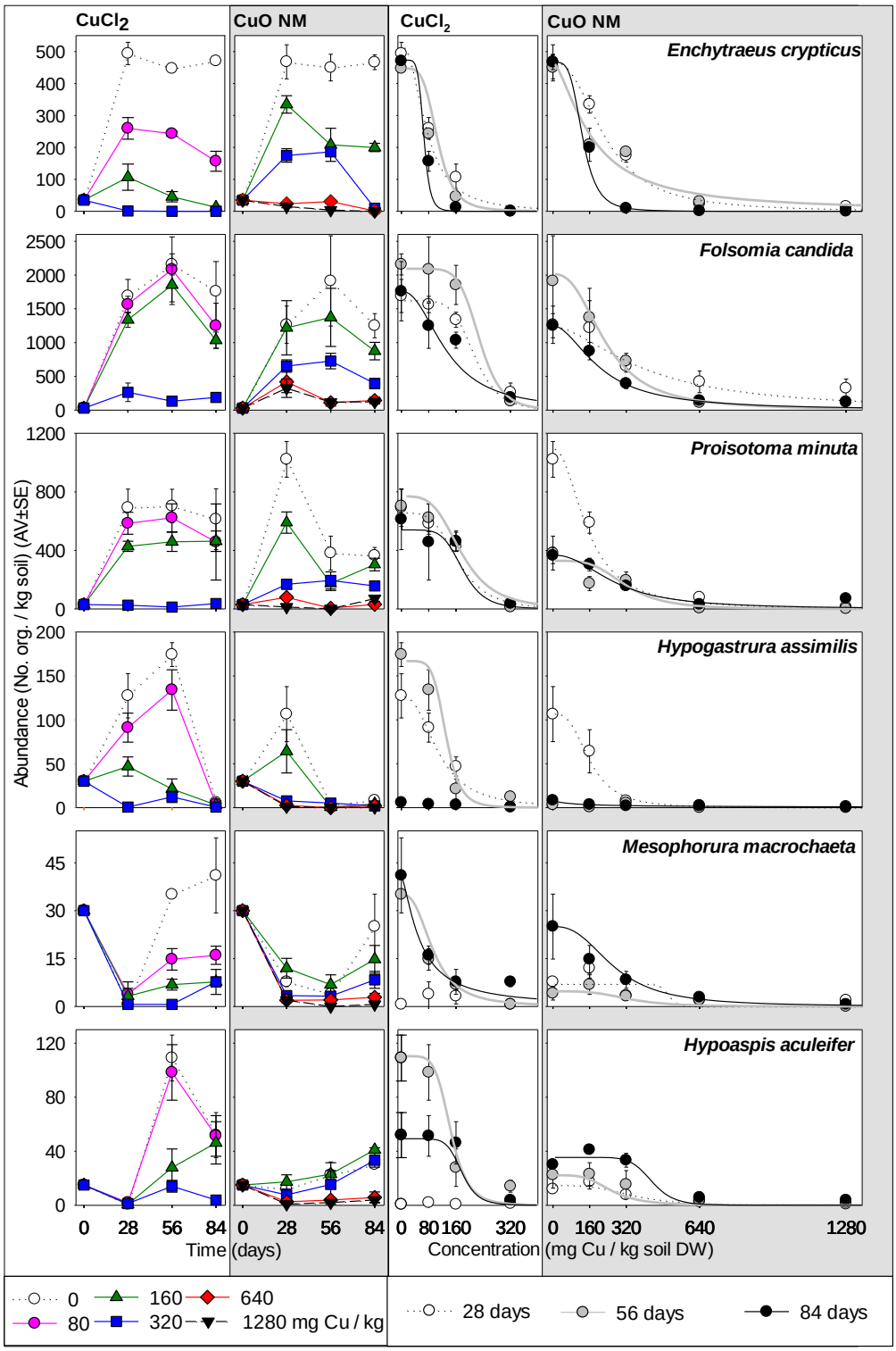

Figure S2: Effects of $CuCl_2$ and CuO NM in the species abundance obtained from a soil multispecies test system experiment performed in LUFA 2.2 soil spiked with CuO NM and $CuCl_2$ and three exposure periods (28, 56 and 84 days). Results are presented as total number of organisms for each species per kg (average ± standard error, n=4). Lines represent the curve fit to model (logistic model with 2 parameters).

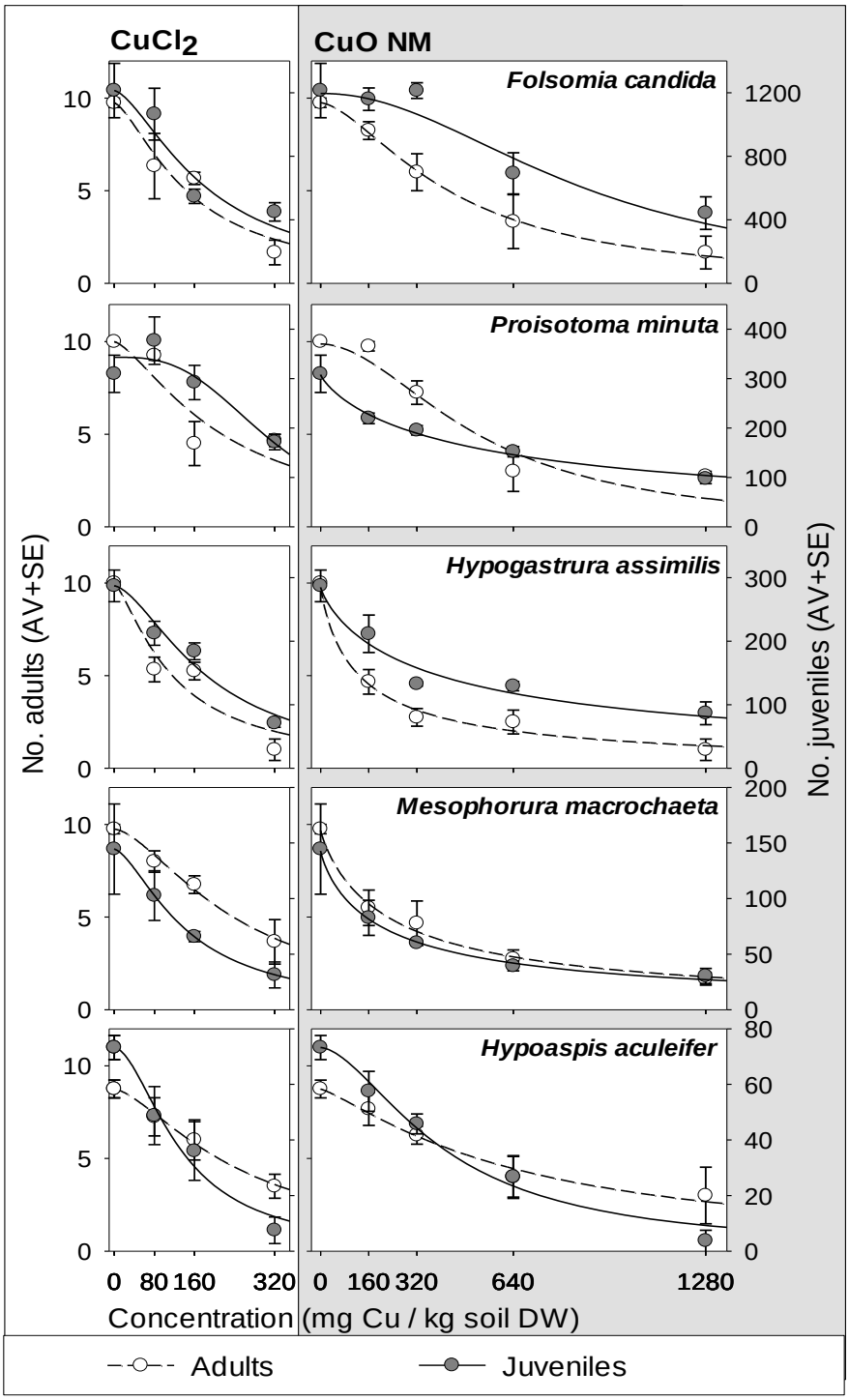

Figure S3: Effects of $CuCl_2$ and CuO NM in terms of number of adults and juveniles obtained in individual species standard testing performed in LUFA 2.2 soil spiked with CuO NM and $CuCl_2$. Results are presented as total number of adults and juveniles (average ± standard error, n=4). Lines represent the curve fit to model (logistic 2 parameters).

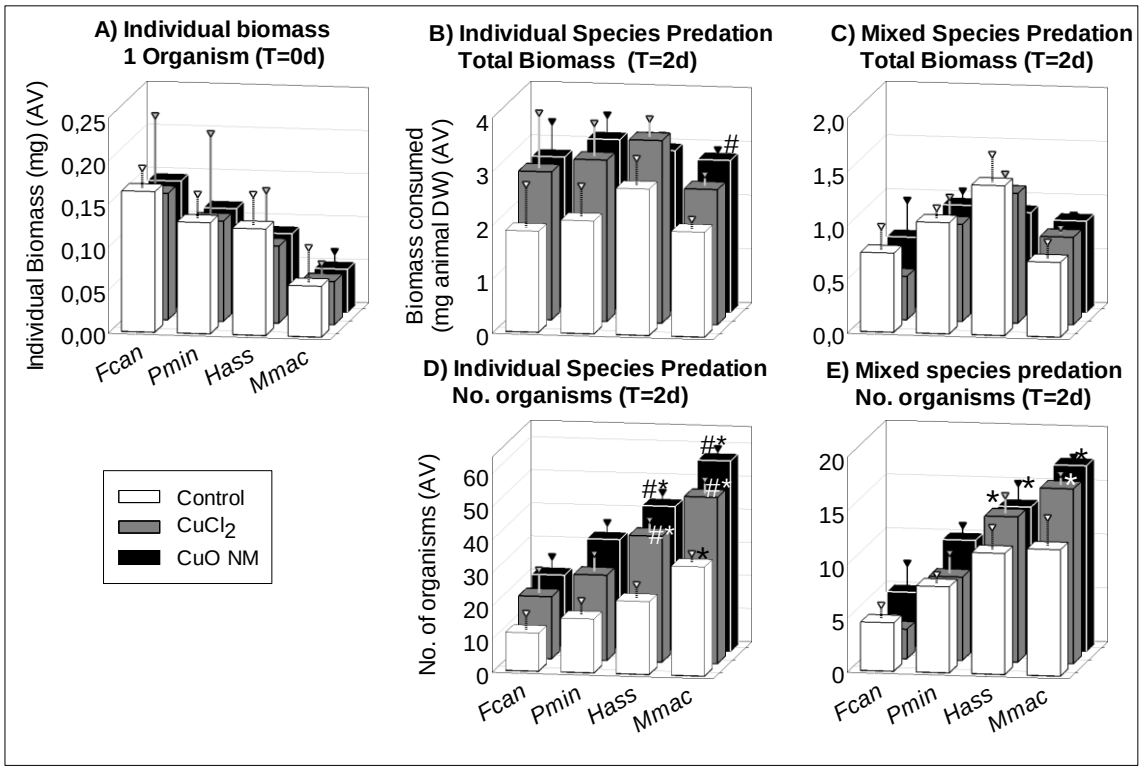

Figure S4: Results of predation test when providing *H. aculeifer* with collembola prey from control and pre-exposed for 7 days to 160 mg Cu/kg DW soil of $CuCl_2$ and 80 mg Cu/kg DW soil of CuO NM. A, B and C as biomass, D and E as number of organisms. All results as average ± standard error (AV±SE). *Fcan: Folsomia candida, Pmin: Proisotoma minuta, Hass: Hypogastrura assimilis, Mmac: Mesophorura macrochaeta*. *($p<0.05$): between species for an individual exposure material. #($p<0.05$): between control and Cu exposed for an individual species.

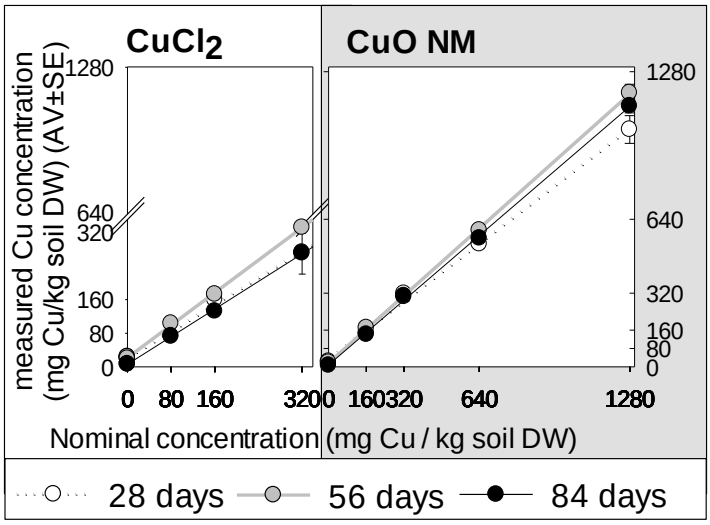

Figure S5: Measured total copper concentrations of the soil multispecies test system (SMS) experiment performed in LUFA 2.2 soil spiked with CuO NM and $CuCl_2$ concentration range of 0, 160, 320, 640 and 1280 for CuO NM; 0, 80, 160 and 320 for $CuCl_2$, and three exposure periods (28, 56 and 84 days). Results presented as mg Cu / kg dry soil (average ± standard error, n=4).

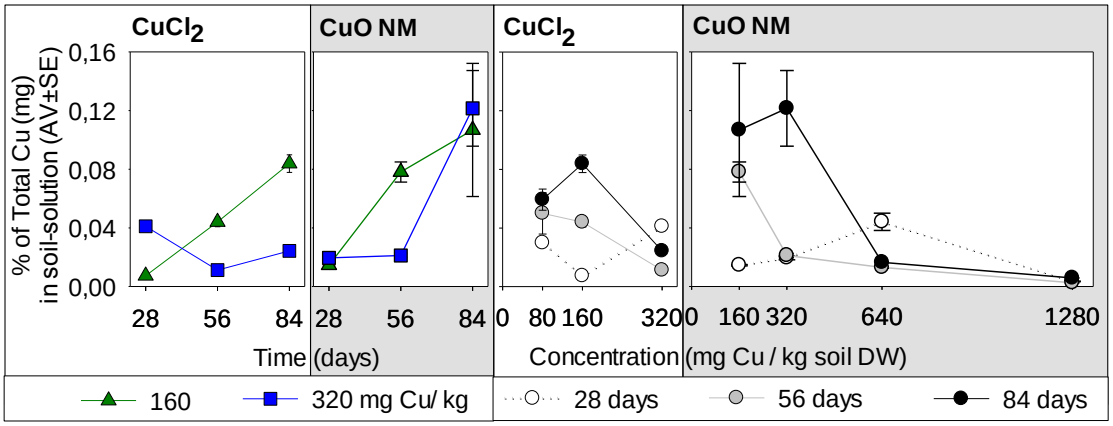

Figure S6: Relative concentration of active total Cu in soil solution of the soil obtained from a soil multispecies (SMS) test system experiment performed in LUFA 2.2 soil spiked with $CuCl_2$ and CuO NM and three exposure periods (28, 56 and 84 days). Results presented as % of total mg Cu (average ± standard error, n=4).

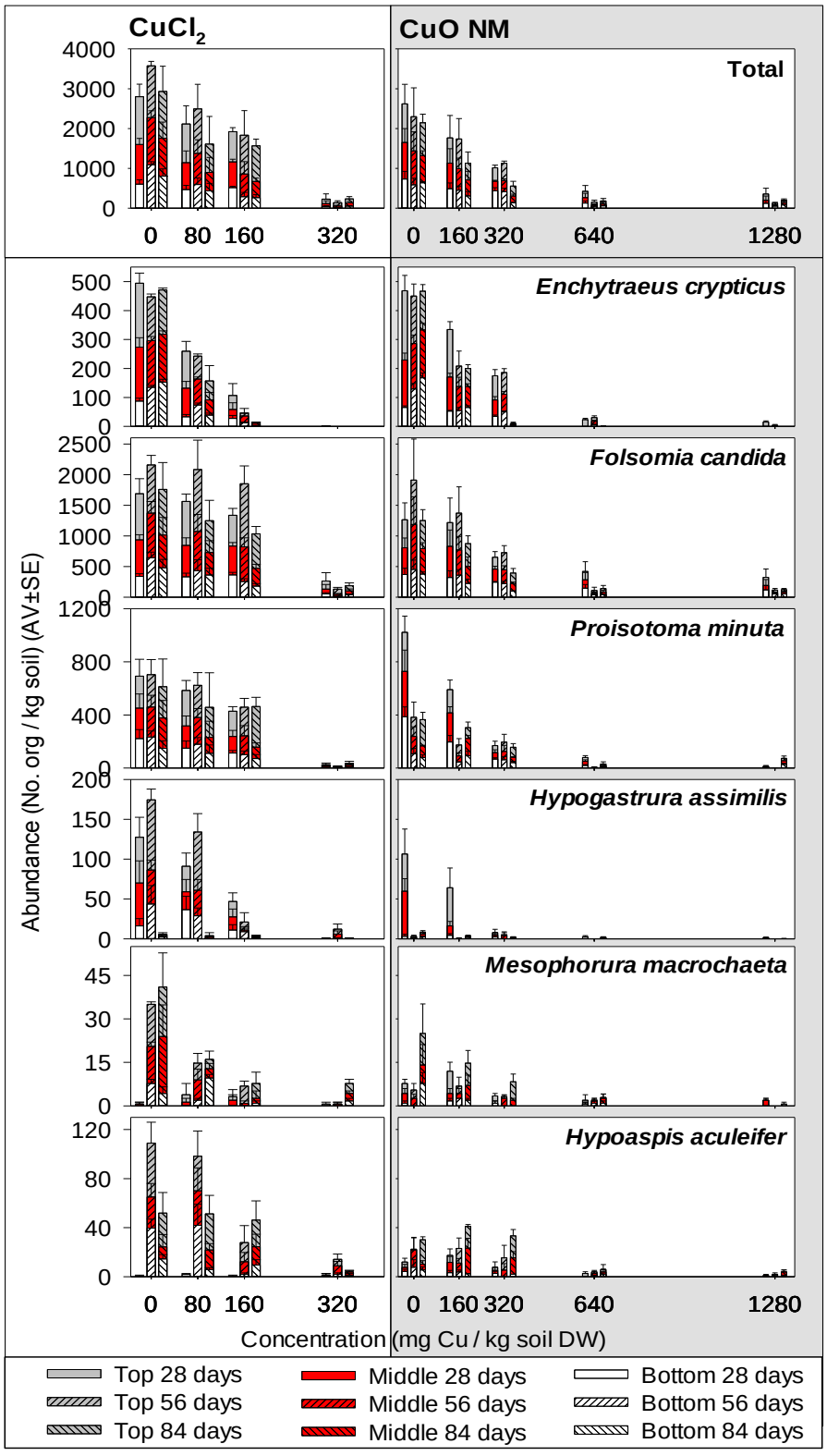

Figure S7: Species abundance layer distribution of the soil multispecies test system (SMS) obtained from a soil multispecies test system experiment performed in LUFA 2.2 soil spiked with $CuCl_2$ and CuO NM and three exposure periods (28, 56 and 84 days). Results presented as number of individuals (average ± standard error, n=4).

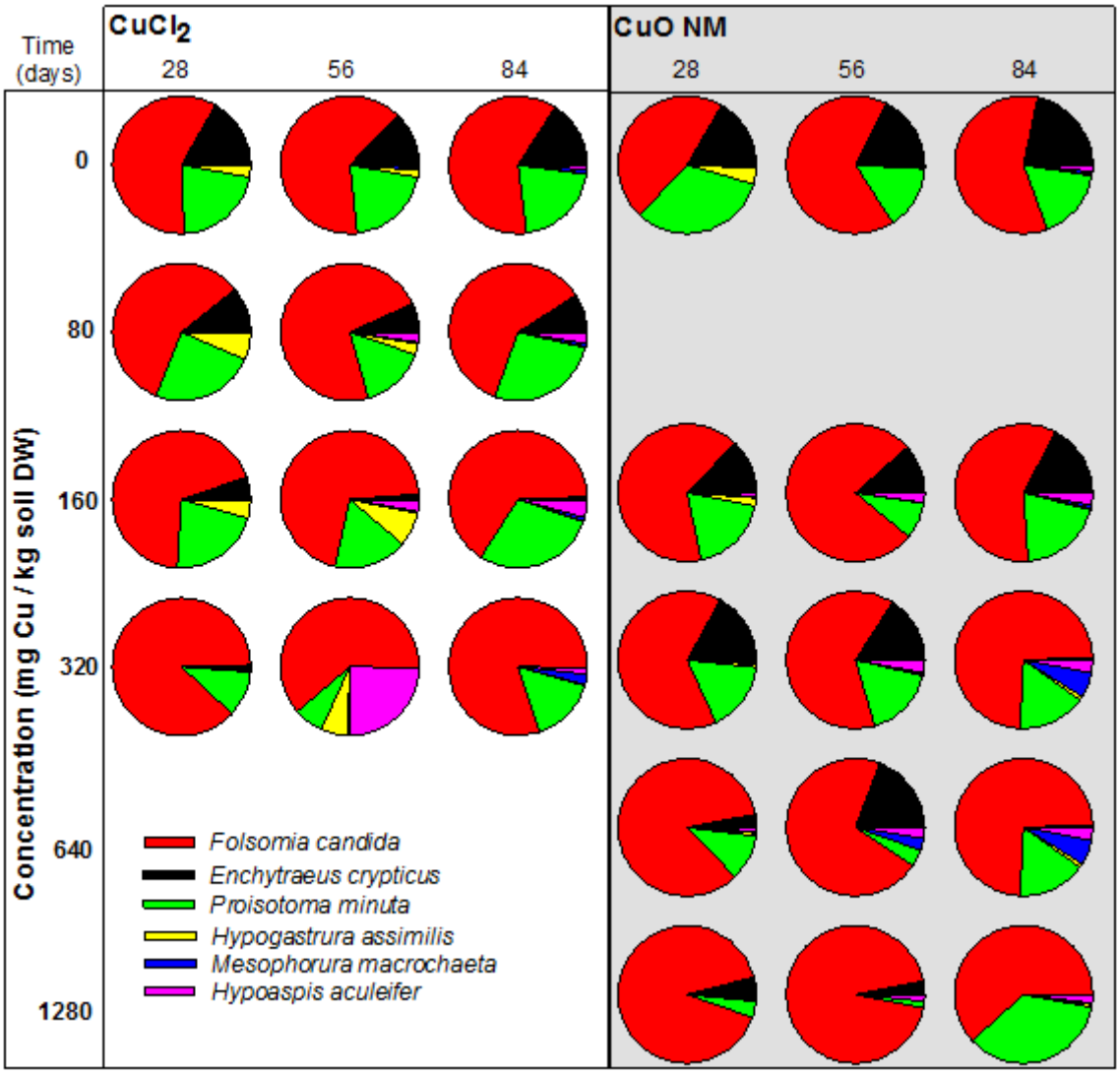

Figure S8: Pie chart with the composition (based on number of organisms) of the soil multispecies test system (SMS) obtained from a soil multispecies test system experiment performed in LUFA 2.2 soil spiked with $CuCl_2$ and CuO NM and three exposure periods (28, 56 and 84 days). Results presented as average % of total population (n=4).

Table S1: Effect Concentrations (ECx) estimates from the soil multispecies test system (SMS) relative to total abundance and individual species in the SMS when exposed to $CuCl_2$ and CuO NM, during 28, 56 and 84 days. The respective model (logistic 2 parameters) parameters are presented. S: slope; Y0: Initial number of organisms. n.d.: not determined; [95% Confidence Intervals].

Endpoint	**Time**	**$CuCl_2$** (mg Cu/kg DW)					**CuO NM** (mg Cu/kg DW)				
Abundance	**(days)**	**EC10**	**EC20**	**EC50**	**EC80**	**Parameters**	**EC10**	**EC20**	**EC50**	**EC80**	**Parameters**
Total abundance	**28**	110 [68-178]	132 [94-183]	178 [150-211]	240 [177-326]	S: 2.65; Y0: 2717	67 [20-228]	108 [45-261]	243 [154-384]	548 [276-1086]	S: 0.98; Y0: 2520
	56	85 [43-165]	109 [68-175]	169 [127-225]	262 [157-436]	S: 1.83; Y0: 3300	115 [46-289]	157 [82-302]	268 [184-389]	456 [243-855]	S: 1.50; Y0: 2421
	84	32 [7-142]	50 [17-142]	105 [65-170]	223 [100-500]	S: 1.06; Y0: 3281	35 [12-105]	60 [27-133]	149 [103-216]	373 [236-589]	S: 0.87; Y0: 2146
Enchytraeus crypticus	**28**	27 [15-50]	39 [25-61]	73 [57-93]	135 [95-191]	S: 1.29; Y0: 518	91 [60-137]	127 [94-172]	227 [190-271]	405 [313-524]	S: 1.38; Y0: 468
	56	66 [58-76]	79 [72-87]	107 [102-112]	145 [133-158]	S: 2.63; Y0: 447	40 [15-106]	68 [33-139]	169 [122-234]	419 [283-621]	S: 0.88; Y0: 467
	84	49 [40-61]	55 [47-64]	67 [63-70]	81 [76-86]	S: 4.16; Y0: 473	77 [55-109]	94 [74-119]	130 [121-140]	182 [157-210]	S: 2.41; Y0: 467
Folsomia candida	**28**	148 [109-201]	169 [137-210]	213 [183-249]	269 [205-353]	S: 3.46; Y0: 1628	109 [25-487]	176 [61-502]	397 [226-695]	894 [333-2400]	S: 0.98; Y0: 1245
	56	169 [114-252]	189 [135-267]	230 [160-329]	279 [170-456]	S: 4.14; Y0: 2093	97 [38-249]	134 [69-261]	231 [162-329]	399 [222-716]	S: 1.46; Y0: 2014
	84	49 [15-159]	71 [31-162]	133 [86-207]	249 [114-546]	S: 1.28; Y0: 1760	65 [30-141]	100 [57-175]	208 [155-278]	434 [289-649]	S: 1.09; Y0: 1250

Proisotoma minuta	**28**	95 [50-181]	117 [74-184]	166 [131-211]	237 [160-349]	S: 2.26; Y0: 659	54 [32-92]	79 [53-117]	150 [123-183]	287 [227-363]	S: 1.23; Y0: 1096
	56	99 [57-170]	122 [83-180]	177 [139-226]	256 [168-388]	S: 2.17; Y0: 769	180 [72-448]	222 [118-417]	318 [221-457]	456 [231-899]	S: 2.22; Y0: 330
	84	123 [45-333]	142 [63-317]	181 [87-376]	230 [83-633]	S: 3.3; Y0: 540	102 [49-210]	146 [85-250]	270 [197-369]	499 [328-760]	S: 1.30; Y0: 368
Hypogastrura assimilis	**28**	45 [21-93]	62 [36-106]	108 [79-149]	189 [117-305]	S: 1.43; Y0: 127	76 [23-259]	101 [42-242]	161 [102-255]	258 [127-524]	S: 1.70; Y0: 107
	56	93 [72-119]	105 [87-128]	131 [113-153]	163 [132-201]	S: 3.65; Y0: 167	n.d.	n.d.	n.d.	n.d.	-
	84	n.d.	n.d.	n.d.	n.d.	-	5 [1-58]	13 [2-89]	77 [28-212]	459 [167-1259]	S: 0.45; Y0: 9
Mesophorura macrochaeta	**28**	n.d.	n.d.	n.d.	n.d.	-	463 [287-749]	478 [350-653]	505 [442-576]	532 [374-757]	S: 14.9; Y0: 7.1
	56	42 [22-80]	57 [36-91]	95 [78-115]	157 [123-202]	S: 1.57; Y0: 35	274 [51-1465]	351 [104-1186]	535 [238-1203]	814 [226-2934]	S: 1.90; Y0: 4.7
	84	12 [1-144]	21 [3-133]	56 [4-130]	148 [62-353]	S: 0.82; Y0: 41	49 [5-448]	80 [16-391]	186 [87-399]	432 [135-1384]	S: 0.95; Y0: 25
Hyposaspis aculeifer	**28**	n.d.	n.d.	n.d.	n.d.	-	182 [52-634]	231 [93-573]	347 [208-579]	520 [246-1098]	S: 1.97; Y0: 15
	56	100 [60-168]	115 [78-170]	146 [117-182]	184 [141-242]	S: 3.38; Y0: 110	212 [54-839]	267 [96-740]	395 [199-749]	586 [214-1606]	S: 2.03; Y0: 22
	84	134 [61-293]	149 [74-297]	178 [82-387]	213 [72-626]	S: 4.46; Y0: 49	273 [175-426]	315 [220-450]	401 [310-518]	510 [385-675]	S: 3.30; Y0: 35

Table S2: Effect Concentrations (ECx) estimates from individual species tests when exposed to $CuCl_2$ and CuO NM. The respective model parameters are presented. The logistic 2 parameters model was used, except when noted (Pcw L: Piercewise linear; Thr: Threshold Sigmoid). S: slope; Y0: Initial number of organisms. n.d.: not determined. [95% Confidence Intervals]. Surv: survival; reprod: reproduction. [a]Bicho et al. (2017).

		$CuCl_2$ (mg Cu/kg DW)					**CuO NM** (mg Cu/kg DW)				
Species	**Endpoint**	**EC10**	**EC20**	**EC50**	**EC80**	**Parameters**	**EC10**	**EC20**	**EC50**	**EC80**	**Parameters**
Enchytraeus crypticus[a]	**Surv**	112 [26-198]	183 [118-247]	303 [251-355]	424 [344-503]	S: 0.003 Y0: 9.3	421 [210-631]	841 [660-1023]	2103 [1855-2352]	3365 [2936-3795]	Pcw L: S: 0.0002 Y0: 8.87
	Reprod	18	78 [45-111]	179 [153-206]	281 [230-332]	S: 0.003 Y0: 598.7	438 [312-616]	643 [504-820]	1377 [1157-1638]	2383 [1773-3202]	Thr:S: 0.0004 Y0: 695.7
Folsomia candida	**Surv**	35 [11-113]	59 [26-134]	150 [103-220]	380 [176-822]	S: 0.86 Y0: 9.75	112 [59-213]	186 [118-292]	440 [335-578]	1043 [645-1687]	S: 0.92 Y0: 9.75
	Reprod	45 [15-136]	76 [36-158]	183 [127-264]	442 [194-1009]	S: 0.90 Y0: 1214	306 [155-603]	452 [286-716]	881 [653-1189]	1716 [949-3105]	S: 1.20 Y0: 1196
Proisotoma minuta	**Surv**	48 [17-132]	83 [43-160]	216 [146-319]	560 [223-1405]	S: 0.84 Y0: 10	167 [102-276]	256 [180-365]	529 [426-656]	1093 [758-1576]	S: 1.10 Y0: 9.88
	Reprod	154 [67-353]	201 [117-345]	318 [230-439]	503 [242-1048]	S: 1.74 Y0: 343	38 [12-124]	103 [48-221]	558 [401-776]	3034 [1260-7304]	S: 0.47 Y0: 310
Hypogastrura assimilis	**Surv**	24 [8-73]	43 [19-95]	117 [84-162]	320 [185-553]	S: 0.79 Y0: 10	10 [2-55]	26 [8-90]	130 [76-222]	650 [402-1049]	S: 0.50 Y0: 10
	Reprod	49 [26-93]	81 [53-124]	188 [150-237]	438 [272-706]	S: 0.95 Y0: 287	28 [6-135]	75 [26-213]	412 [272-622]	2267 [774-6634]	S: 0.47 Y0: 287
Mesophorura macrochaeta	**Surv**	63 [32-121]	104 [68-158]	246 [188-321]	583 [313-1086]	S: 0.92 Y0: 9.75	20 [4-95]	50 [17-149]	237 [155-363]	1126 [541-2343]	S: 0.51 Y0: 9.75
	Reprod	36 [5-276]	60 [15-243]	143 [76-269]	344 [97-1216]	S: 0.91 Y0: 145	15 [1-839]	42 [3-685]	219 [77-621]	1157 [208-6450]	S: 0.48 Y0: 145
Hypoaspis aculeifer	**Surv**	59 [22-160]	101 [53-191]	252 [170-375]	632 [250-1595]	S: 0.87 Y0: 8.75	111 [38-322]	215 [107-432]	659 [434-999]	2020 [813-5019]	S: 0.71 Y0: 8.75
	Reprod	40 [14-112]	62 [30-128]	133 [92-192]	286 [150-546]	S: 1. 04 Y0: 73	113 [62-206]	182 [120-279]	411 [323-524]	928 [614-1401]	S: 0.98 Y0: 73

Chapter five

Zeta Potential to Predict Nanomaterials Toxicity and Safety by Design - On Surface Modified CuO NMs Using a Soil Multispecies Test System

Zeta Potential to Predict Nanomaterials Toxicity and Safety by Design - On Surface Modified CuO NMs Using a Soil Multispecies Test System

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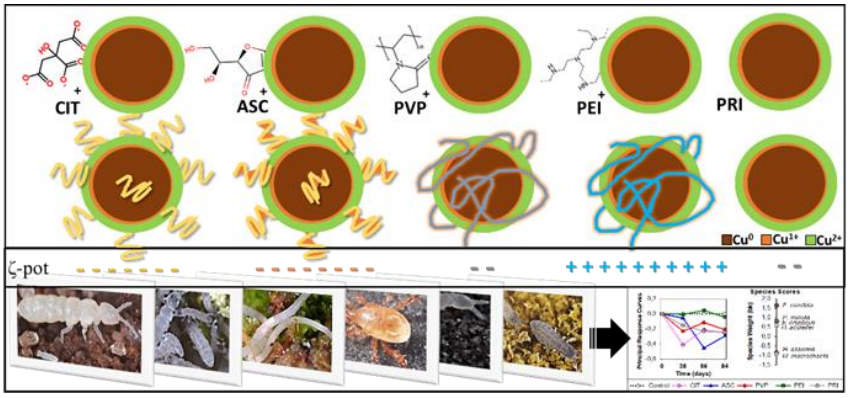


Abstract

Safer by design modifications of nanomaterials (NMs) has been pursued since these can keep functionality and yet reduce hazard and support sustainable nanotechnology. The present case study involves copper oxide nanomaterials (CuO NMs) based paint. These CuO NMs were surface modified by a "Safety by Design" (SbyD) approach to obtain particles coated with sodium citrate (CIT^-), sodium ascorbate (ASC^-), polyethylenimine (PEI^+), and polyvinylpyrrolidone (PVP). Hence, in this study we assessed the effect of the 4 different surface modified (CIT, ASC, PVP and PEI) plus the pristine non-coated (PRI), using the soil multispecies test system. The total test duration was 84 days, (samples at 28−56−84 days). Further, the species were tested individually, Cu was measured in test media (soil and soil solution) and organisms. Ecotoxicity differed between the various surface modified CuO NMs: –CIT and –ASC were more toxic than the other (-PEI << -PVP ≤ Pristine), which correlated with zeta potential: -PEI (+28mV) caused the least impact, -ASC and -CIT (-17mV, -18mV) the most, while PVP and PRI

(-8mV, -9mV) caused an intermediate response. The observed differences were not explained by the contribution of soluble Cu. Coating interfered with the release of Cu^{2+} and/or the activation of copper regulators and detoxification mechanisms in the organisms, i.e. time to reach steady state was faster (-ASC) and slower (-PVP) during prolonged time. The current risk assessment framework should move towards longer term testing and cover multispecies approaches (at least increase the number of mandatory individual species testing required).

Keywords: surface modification; coating; safe-by-design; read-across; mesocosms; long-term exposure; realistic scenarios; ecosystem function;

1. Introduction

The number of products containing nanomaterials (NMs) continues to increase, the global nanotechnology market is expected to grow up to US$ 75.8 Billion until 2020. Hence there is a concern regarding the associated risks of NMs during the various life cycle stages towards non target species. The increased use NMs makes them a significant portion reaching the environment, e.g. via waste activated sludge (Ünşar et al., 2016): Around 40-53% of the sludge is used in agriculture fields in the USA, Canada and Europe (Ünşar et al., 2016). Research focused on safer by design modifications of NMs has been given attention. While keeping NMs functionality, the aim is to reduce hazard and ensure a sustainable nanotechnology industry.

Copper oxide nanomaterials (CuO NMs) are widely used, among other, as antimicrobial coatings, antifouling paints, catalysis, superconducting, thermoelectric, sensing materials, wood preservative and fertilizers (Evans et al., 2008; Pang et al., 2012; Ramskov et al., 2014). Safe by design approaches have tested Fe doped Cu to tune Cu dissolution rates (Naatz et al., 2017). The present case study involves a CuO NMs based paint, which provides aesthetic functionality to softwood cladding besides wood preservation. The application process includes wood saturation and the remaining copper precipitates as basic copper carbonate, which can be released into the environment through leaching. Here, CuO NMs were surface modified by a “Safety by Design” (SbyD) approach (Petros and DeSimone, 2010; Gardini et al., 2013; Ortelli and Costa, 2018) to include preferably non-hazardous modifying agents and obtain different surface charges: sodium citrate (CIT^-), sodium ascorbate (ASC^-), polyethylenimine

(PEI^{+}), and polyvinylpyrrolidone (PVP) (Ortelli et al., 2017). ASC and CIT have antioxidant properties and can be used as negatively charged stabilizers (Bastos et al., 2016, 2017); PEI is a synthetic cationic polymer which provides electrosteric stabilization when attached to NMs surfaces (Xia et al., 2009); PVP is a neutral polymer with high hydrophilic component (pyrrolidone moiety) and a hydrophobic group (alkyl) used both as dispersing and shape-control agent (Koczkur et al., 2015)..

Cytotoxicity of CuO NMs (-CIT, -ASC, -PEI, -PVP) in mouse cell lines (Líbalová et al., 2018) indicated synergistic interactions between the NMs, dissolution rate and the toxicity of the coating agents but no clear relationship with the materials properties. The ecotoxicity of the pristine CuO NMs (non-coated, as synthesized) has been studied on soil invertebrates (Alahdadi and Behboudi, 2015; Bicho et al., 2017a, 2017b; Gonçalves et al., 2017; Mendes et al., 2018) but not the surface modified CuO NMs. Hence, in this study we assessed the effect of the 4 different surface modified (CIT, ASC, PVP and PEI), using the soil multispecies test system (Scott-Fordsmand et al., 2008). The multispecies consist of six species (one enchytraeid, one mite, four collembolans, and microbes). The total test duration was 84 days, with intermediate samples at 28−56−84 days. To support interpretation, the species were also tested individually, Cu was measured in test media (soil and soil solution) and organisms.

2. Material and Methods

2.1. Soil Multispecies Test System (SMS)

The soil multispecies system (SMS) was prepared based on the previously optimized by Scott-Fordsmand et al. (2008). Briefly, the species composition of the SMS represents an agro-ecosystem with six species belonging to different functional groups with different interactions represented (mutualism, competition, predation, etc.). The study was performed using a full factorial design with four surface modified CuO NMs (-PVP, -PEI, -CIT, -ASC), two exposure concentrations and three exposure durations. In total 60 SMS units (replicates) were prepared.

2.2. Test Soil and Spiking

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The main characteristics can be described as follows: pH (0.01 M $CaCl_2$) = 5.5, organic matter = 1.77%, CEC (cation exchange capacity) = 10.1 meq/100 g, WHC (water holding capacity) = 41.8 %, grain size distribution of 7.3 % clay, 13.8 % silt, and 78.9 % sand. Soil was first defaunated by drying at 80°C for 48h.

The materials were tested at 0 and 200 mg Cu / kg soil dry weight (DW) for all the CuO-surface modified NMs. The concentration was selected based on the reproduction EC50 as obtained in Mendes et al. (2018).

Soil spiking with surface modified CuO NMs followed the recommendations for nanomaterials in solution (OECD, 2012; Hund-Rinke et al., 2016). In short, surface modified CuO NMs aqueous solution was added to each pre-moistened soil replicate individually to ensure equal spiking, and mixed thoroughly to homogenize. The pre-moistening was done with an aqueous microbial substrate in a volume of 10% (v/w). Soil moisture was adjusted to 40% of the maximum water holding capacity (WHC). Soil was equilibrated for 3 days prior to test start

2.3. Microbial Substrate

The microbial substrate was obtained, following Scott-Fordsmand et al. (2008). In short, 1kg of LUFA 2.2 soil was shaken for 3 h with 2l of deionized water, sieved through a 50 μm mesh and diluted 10 times before use. The aqueous substrate was added to the soil to start microbial activity, 3 days prior test start.

2.4. Test Materials and Characterization

Copper oxide nanomaterials (CuO NMs) as synthesized (results from Mendes et al. (2018)) and with four different coatings were tested: citrate (CIT), ascorbate (ASC), polyvinylpyrrolidone (PVP) and polyethylenimine (PEI). Materials were synthetized from commercial CuO nanopowder (PlasmaChem GmbH, Germany), and prepared according to Ortelli et al. (Ortelli et al., 2017). Morphological characterization of pristine CuO NPs by STEM analysis showed that CuO NPs are spherical and mono-dispersed with a primary nanoparticle average diameter of 12 ± 8 nm (N=50), see table S1 for full characterization details.

In short, a CuO NMs stock solution of 10 g Cu / L was prepared by dispersing the CuO NMs powder in a Phosphate Buffer Solution, and from this stock solution, modified CuO NMs dispersions were prepared with sodium citrate (CIT), sodium ascorbate (ASC), polyvinylpyrrolidone (PVP) and polyethylenimine (PEI), guaranteeing a complete covering of CuO NMs. The surface modified CuO NMs characterization as dispersed in water are presented in Table 1.

Copper was measured in the soil, soil solution and organisms by Graphite Furnace Atomic Absorption Spectroscopy (AAS-GF) (Perkin Elmer 4100, Ueberlingen, Germany), following the method details as in Gomes et al. (2015), at sampling days 28, 56 and 84. The free active Cu was measured by ion-selective electrode (ISE25Cu-9) with REF251 reference electrode (Radiometer Analytical, Lyon, France).

In short, for Cu measurements by AAS-GF, samples were acid digested (HNO3 65%) for 3-7 days. A reference tissue for copper concentration was used (National Institute of Standards and Technology, 1989).

The CuO present as nanomaterials was not determined in the soil, due to the technical issues i.e. the particle size is below the theoretical detection limit of 15 nm (Navratilova et al., 2015).

For the measurements in organisms from the SMS, only *F. candida* was analysed due to issues of sufficient biomass from individual replicates and extraction; for individual species testing both *F. candida* and *P. minuta* were analysed. In the SMS, at each sampling time 10 *F. candida* were obtained from the top layer of each unit by flotation in distilled water, retrieved, transferred into a 1.5 ml Eppendorf tube and snap frozen in liquid nitrogen. Before the acid digestion step, animal samples were unfrozen and dried at 80°C for 24h to obtain the samples dry weight. In the individual species testing the same procedure was followed but keeping all the organisms instead of 10.

2.5. Test Organisms

Six species were included: four collembolans (*Folsomia candida, Proisotoma minuta, Hypogastrura assimilis* and *Mesophorura macrochaeta*), one predatory mite (*Hypoaspis aculeifer*) and one enchytraeid (*Enchytraeus crypticus*). All organisms are from well-characterised populations, cultured in the laboratory for several years at DMU. The initial number per unit was 30 for each collembolan species, 15 for the mites and 50

enchytraeids, consisting of random sized adult animals, all added at test start (day 0), except for *H. aculeifer*, added at day 7. For details see Mendes et al. (2018).

2.6. SMS Units' Experimental Conditions

The experimental SMS units consisted of polyethylene tubes (33 cm × 9.3 cm ø), having a surface area of 68 cm^2 and sample volume of 2241 cm^3. The tubes, closed on the bottom with a perforated lid, were filled with 1000 g moist soil (800 g dry weight plus 100 ml CuO NMs solution and 100 ml aqueous microbial substrate). Experiments were performed in temperature controlled rooms (20±1°C) with 12:12h light:dark cycle. The soil water content was maintained by replenishing water loss weekly.

2.7. Sampling and Extraction

At each sampling time (28, 56 and 84 days), 4 SMS units (replicates) for each concentration and material were extracted. The soil of each replicate was divided in 3 equal sized layers (top, middle and bottom) with approximately 225g dry weight and 5 cm height. From each layer ca. 100g (random sub-samples) were used to quantify arthropods, ca. 45 g to quantify enchytraeids, 10g to quantify Cu in soil and soil solution. The microarthropod sub-samples were extracted over 3 days in a MacFadyen high gradient extractor (Scott-Fordsmand et al., 2000) with temperature rising from 30 to 60°C. Animals were collected in benzoic acid, transferred to glycerol, identified and counted. The animals for Cu analyses were sampled alive by floatation of subsample. The enchytraeids were extracted by spreading the soil sample into four 200 ml plastic beakers (ø 7 cm), filled with tap water and ethanol, gently shaken, and then left for 24 h at 5°C for sedimentation. Afterwards samples were washed and sieved in a 50 µm mesh to extract the enchytraeids for counting.

2.8. Individual Species Tests

Individual species tests were carried out for the collembolans *F. candida*, *P. minuta* and *H. assimilis*, following the standard guideline for *F. candida* (OECD, 2009) with adaptations. *F. candida* 10-12 days old synchronized juveniles were used. *P. minuta* and *H. assimilis* juveniles were obtained from established cultures with similar sizes but not synchronized age, resembling the animals selected for the SMS. All tests ran for 28 days. The concentration ranged between 0-1000 mg Cu/kg soil DW and were selected

for each CuO-surface modified NM based on preliminary tests and previous results on CuO NMs (Mendes et al., 2018) as indicated in table 1.

Table 1: CuO NMs individual species tests concentration range, presented as mg Cu / kg soil dry weight (DW). Test species: *Folsomia candida*, *Proisotoma minuta* and *Hypogastrura assimilis*. CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine; PRI: Pristine (from Mendes et al. (2018).

CuO NMs surface modified	CuO NMs (mg Cu / kg soil DW)		
	F. candida	*P. minuta*	*H. assimilis*
CIT	0-75-125-200-400	0-50-100-175-250	0-40-80-120-180
ASC	0-75-125-200-400	0-50-100-175-250	0-40-80-120-180
PVP	0-200-400-600-1000	0-100-200-350-500	0-75-150-250-400
PEI	0-100-150-225-400	0-70-120-200-300	0-50-100-175-250
PRI	0-160-320-640-1280	0-160-320-640-1280	0-160-320-640-1280

2.9. Data Analysis

Multivariate: For the SMS experiment, abundance data was analyzed using principal response curves (PRC) based on the redundancy analysis (RDA) (van den Brink et al., 2009; van den Brink and Ter Braak, 1999) using the software package CANOCO version 4.5 (ter Braak and Smilauer, 2002). The significance of the PRC diagrams was tested by Monte Carlo permutation tests by permuting whole time series in the partial RDA, from which the PRC was obtained. All data were ln transformed prior analyses.

Univariate: All samples were tested for normality (Shapiro-Wilke). One-way ANOVA was used to assess differences between treatments for population data (abundance, survival and reproduction) and copper concentration data. Post-Hoc Dunnetts' test and Tukey's test were used to assess differences between control and exposure for each sampling time and differences between time points for the same exposure, respectively (Sigmaplot, 1997). For effect concentrations (ECx) estimates the logistic 2 parameters model was used, using the Toxicity Relationship Analysis Program software (TRAP 1.30) (Erickson, 2015).

Data from pristine CuO NMs testing from previous dose response SMS study (Mendes et al., 2018) was integrated by normalizing it to the current control data for direct comparison.

3. Results

3.1. Soil Multispecies System

The pH did not vary significantly with treatment or exposure time, being ca. 6.5±0.01.

Overall, population growth curve (Figure S1) for control showed an increase from 0 to 28 days, followed by a plateau (28-56d) and a slight decrease (56-84d). The same pattern was observed for all the surface modified CuO NMs, although with lower total number, especially after 84 days.

Clearly the individual species were affected differently with exposure time and material, and the relative numbers are distinct (Fig.S1). For instance, *E. crypticus* showed the highest toxicity between control and CuO NMs (except with PEI-) as opposed to *H. assimilis*, and *H. aculeifer* showed the opposite pattern with an increase in numbers at later exposure periods (84d).

The multivariate analysis (RDA) estimates 90% of the total variation could be explained: 8% being explained by time, 86% by the NM and 4% by both time and NM.

The PRC display (Figure 1) showed -PEI with the least and –ASC/-CIT with the highest ecosystem impact. The species most influencing the result were in opposite sides: *F. candida* (high numbers) versus *M. macrochaeta* and *H. assimilis* (low numbers).

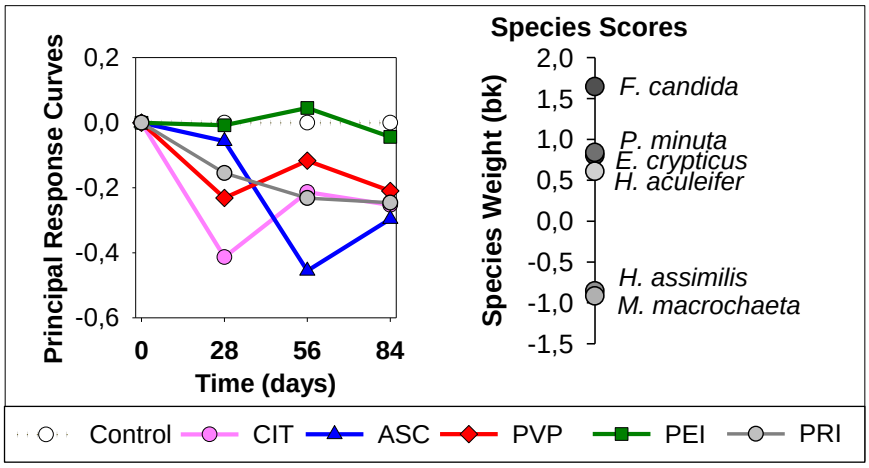


Figure 1: Principal response curves (PRC) with species scores (bk), obtained from the soil multispecies test system (SMS) experiment performed in LUFA 2.2 soil spiked with surface modified CuO NMs (0-200mg CuO NMs/kg soil DW) during three exposure periods (28, 56 and 84 days). CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine. PRI: Pristine, not surface modified.

The Monte-Carlo permutation test confirmed significant differences ($p<0.05$) between control and all, except PEI-surface modified NMs and 56 days PRI (Table S2).

3.2. Individual Species Testing

The individual species tests fulfilled the validity criteria as in standard guideline for *F. candida* (OECD, 2009). The pH was 5.9±0.1 in control (without significant variation with time) and in CuO NMs spiked soil pH varied between 5.9 to 6.4 from day 0 to 28.

Results showed variations for species sensitivity and materials (Figure 2). For instance, for *P. minuta* the effect on adult survival was more pronounced in all except PEI- whereas there was little to no effect for *F. candida* survival.

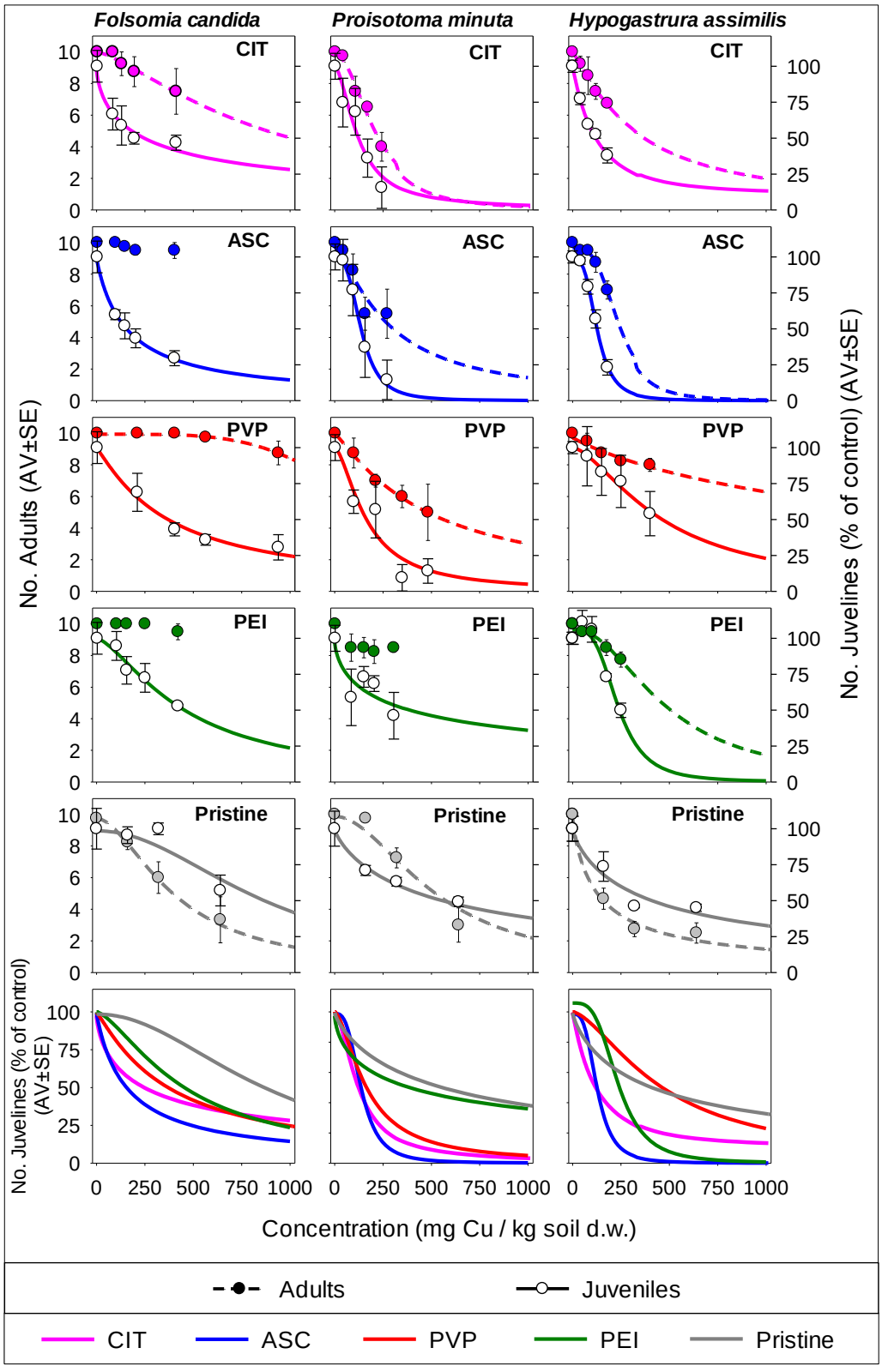


Figure 2: Results in terms of survival and reproduction obtained in individual species standard testing performed in LUFA 2.2 soil spiked with CuO NMs. Results are expressed as average ± standard error (AV±SE, n=4). Lines represent the curve fit to model (logistic 2 parameters). CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine.

The effect concentrations were estimated and summarised in Table 2. In terms of reproduction, a dose response was obtained for all surface modified CuO NMs within the tested range. Overall, PEI and PVP were the least toxic.

Table 2: Effect Concentrations (ECx) estimates from results obtained in the individual species tests when exposed to different CuO NMs surface modified. The ECx (mg Cu / kg soil DW) are presented with the respective model parameters. The logistic 2 parameters model was used. S: slope; Y0: Initial number of individuals. n.e.: no effect; [95% Confidence Intervals]. CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine; PRI: Pristine.

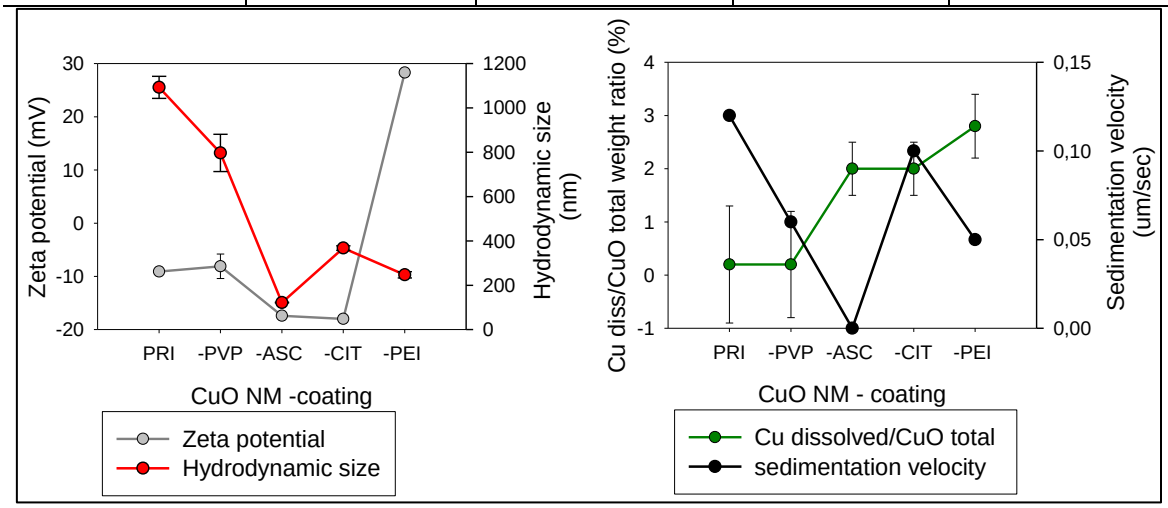
CuO NMs	Survival			Reproduction		
-coating	EC10	EC50	Parameters	EC10	EC50	Parameters
Folsomia candida						
-CIT	174 [91-334]	603 [307-1185]	S: 1.02 Y0: 10.0	9 [1-112]	247 [126-486]	S: 0.39 Y0: 397
-ASC	n.e.	n.e.	-	16 [4-70]	156 [113-217]	S: 0.55 Y0: 397
-PVP	714 [466-1095]	1597 [665-3842]	S: 0.25 Y0: 10	52 [12-233]	370 [257-534]	S: 0.65 Y0: 397
-PEI	n.e.	n.e.	-	104 [46-236]	455 [280-738]	S: 0.86 Y0: 397
PRI	112 [59-213]	440 [335-578]	S: 0.92 Y0: 10	306 [155-603]	881 [653-1189]	S: 1.20 Y0: 397
Proisotoma minuta						
CIT	72 [44-116]	210 [176-252]	S: 1.18 Y0: 9.9	35 [10-125]	124 [81-190]	S: 1.00 Y0: 253
ASC	51 [17-153]	270 [156-469]	S: 0.76 Y0: 9.8	66 [31-141]	137 [100-188]	S: 1.74 Y0: 253
PVP	101 [31-325]	518 [300-894]	S: 0.77 Y0: 9.4	36 [8-169]	172 [106-280]	S: 0.82 Y0: 253
PEI	n.e.	n.e.	-	29 [4-201]	577 [170-1963]	S: 0.42 Y0: 253
Pristine	167 [102-276]	529 [426-656]	S: 1.10 Y0: 9.9	38 [12-124]	558 [401-776]	S: 0.47 Y0: 253
Hypogastrura assimilis						
CIT	52 [24-114]	324 [148-708]	S: 0.69 Y0: 10	17 [10-30]	122 [104-142]	S: 0.65 Y0: 243
ASC	125 [97-160]	242 [182-321]	S: 1.91 Y0: 9.7	67 [54-82]	129 [119-141]	S: 1.90 Y0: 239
PVP	171 [103-286]	1938 [424-8852]	S: 0.43 Y0: 10	116 [28-487]	465 [194-1113]	S: 0.91 Y0: 243
PEI	169 [126-226]	479 [245-935]	S: 1.21 Y0: 10	124 [90-172]	237 [209-269]	S: 1.97 Y0: 257
Pristine	10 [2-55]	130 [76-222]	S: 0.50 Y0: 10	28 [6-135]	412 [272-622]	S: 0.47 Y0: 243

3.3. In situ Characterization

The characterization of dispersed surface modified CuO NMs results in miliQ water, was summarized in Table 3.

Table 3: Characterization of pristine and surface modified CuO NMs samples dispersed in Milli-Q water (pH = 6.5), including ζ-potentials (mV), hydrodynamic diameter (nm), sedimentation, velocity (µm /sec), $Cu_{dissolved}/CuO_{total}$ weight ratio (%) after 24 h at 25 °C (from Ortelli et al. (2017). CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine; PRI: Pristine. The reversal of the CuO pristine surface charge sign is due to the presence of phosphate ions (PO_4^{3-}) used in the sample preparation, which are specifically adsorbed onto the CuO NMs surface. Graphs below show the data plot.

	ζ-potential (mV)	hydrodynamic diameter (nm)	sedimentation velocity (µm /sec)	$Cu_{dissolved}/CuO_{total}$ weight ratio (%)
CuO-PRI (PO_4^{3-})	−9.1 ± 0.4	1093 ± 50	0.12	0.2 (1.1)
CuO-PVP	−8.1 ± 2.3	797 ± 84	0.06	0.2 (1.0)
CuO-ASC	−17.4 ± 0.3	122 ± 1.4	0.0	2 (0.5)
CuO-CIT	−18.0 ± 0.3	368 ± 10	0.1	2 (0.5)
CuO-PEI	+28.3 ± 0.7	247 ± 14	0.05	2.8 (0.6)



The characterization of Cu in the test media – soil – showed a that the total Cu measured in the soil was ca. 99.3 ± 0.1% and 104.9 ± 1.8% (average ± standard error) of the nominal total added concentration, in the SMS and in the individual species tests respectively (Fig. S4 for details). The % of total Cu in soil-solution was ca. 1%, with no significant difference between surface modifiers and with exposure time, except for PEI-CuO NMs, where an increase to 2.5±1.6% was measured after 84 days. The % of

active Cu in soil-solution was below 0.01%, except for PVP at 28 days with 0.02 ± 0.01% (Figure 3).

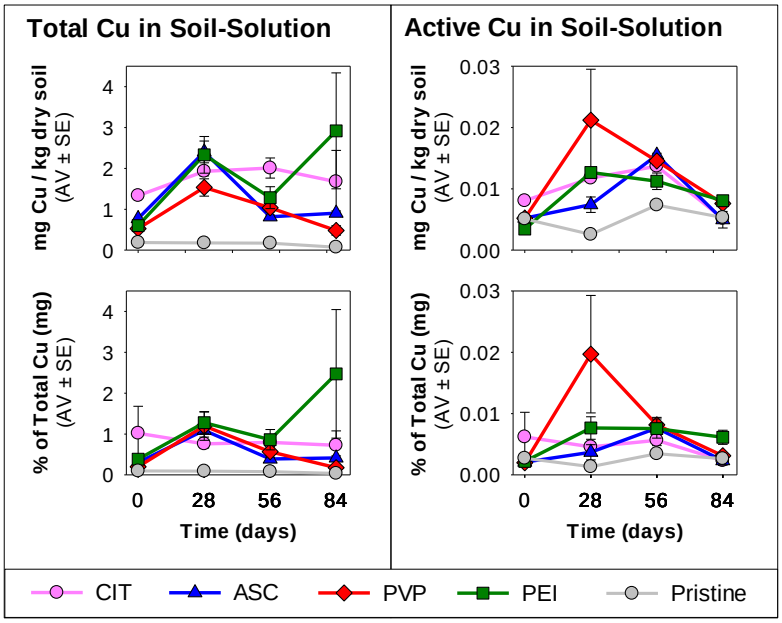


Figure 3: Total and active Cu in soil solution, obtained from the soil multispecies test system (SMS) experiment performed in LUFA 2.2 soil spiked with surface modified CuO NMs (0-200 mg CuO NMs/ kg soil DW) and three exposure periods (28, 56 and 84 days). Results are expressed as average ± standard error (AV±SE, n=4). CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine; PRI: Pristine.

Measurements of body Cu concentration in *F. candida* from the SMS experiment (Fig.4A) showed that CIT- was close to control values, i.e. little accumulation. The accumulation was higher for ASC-, PVP- and PEI-, with maximum values of ca. 250, 240, 170 µg Cu/g organism DW (mg Cu/kg equivalent) respectively. The steepest increase was up to 28 days, after which PVP- continued to slowly increase until 84 days whereas the other reached a plateau.

Body Cu concentration in *F. candida* from the individual species experiment (Fig. 4B) showed that uptake increased with exposure concentration during the 28 days. Maximum uptake concentrations must be around 250 µg Cu/g organism DW as observed by the maximum value reached for 1000mg Cu/kg soil DW exposure for PVP-CuO NMs. Comparatively, *P. minuta* accumulated less than *F. candida*.

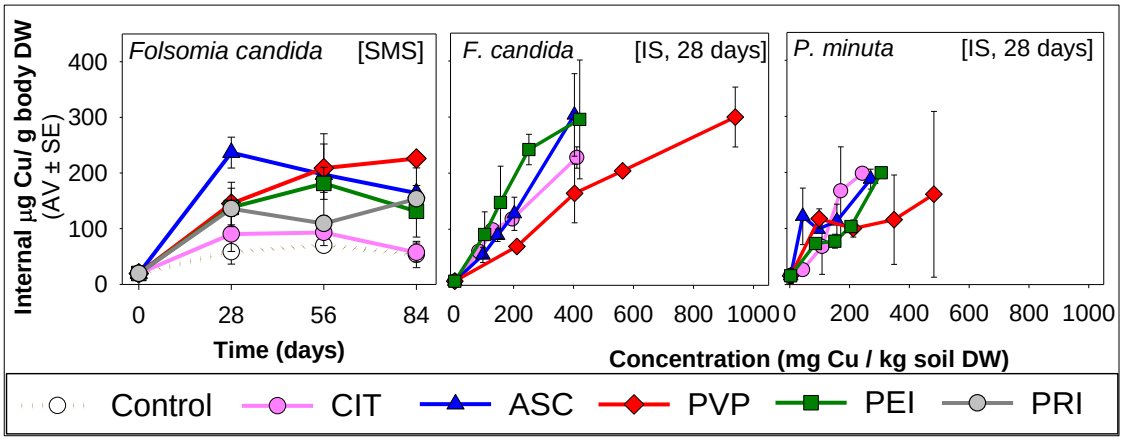


Figure 4: Total internal copper concentration in *F. candida* obtained from the soil multispecies test system (SMS) experiment performed in LUFA 2.2 soil spiked with CuO NMs surface modified (0-200 mg CuO NMs/ kg soil DW) and three exposure periods (28, 56 and 84 days) and in *F. candida* and *P. minuta*, obtained from individual species tests exposed during 28d. Results are expressed as µg Cu / g body DW (average ± standard error, n=4). CuO NMs coating: CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine; PRI: Pristine.

4. Discussion

Results from the SMS showed power to discriminate between various surface modified CuO NMs, positioning –CIT and –ASC as relatively more toxic than the other (-PEI << -PVP ≤ PRI). There was a correlation between zeta potential and effect: -PEI (+28mV) caused the least impact, -ASC and -CIT (-17, -18mV) the most, while PVP and PRI (-8, -9mV) caused an intermediate response. The surface modified CuO NMs charge (+28 ≤ ζ-pot ≤ -17) improved colloidal dispersion, i.e. decreased hydrodynamic size compared to the pristine and PVP. It has been suggested that this could be due to the long aliphatic chains that probably force NP aggregation by depletion flocculation (Ortelli et al., 2017). This is known to depend on the dispersion media or surrounding matrix, and aggregation will occur in several composed salt media, where e.g. there is increase of ionic strength due to the salt content. CuO NMs-PEI was the best dispersed system in both Milli-Q water and in a high ionic strength medium (phosphate buffered saline (PBS)) and authors (Ortelli et al., 2017) note the additional electrosteric action of polyethylenimine: -ASC- and -CIT salts transfer the negative charge to the particle surface, improving the repulsive potential, -PEI provides both electrostatic (due to its positive charge) and steric contributions, due to its polymeric structure. This must be

somehow associated with the lowest toxicity caused by –PEI (and highest in –ASC and –CIT). CuO-PRI and CuO-PVP have ζ-pot close to zero, indicating proximity to the isoelectric point (pHIEP) and the biological response (intermediate) was similar between -PVP and –PRI.

In terms of the solubility of dispersed Cu, it seemed to be size-dependent (in Milli-Q and PBS): the low aggregation rate corresponded to higher solubility (CuO-CIT and CuO-PEI) and, the more aggregated (CuO-pristine, CuO-PVP and CuO-ASC) showed lower values of the $Cu_{dissolved}/CuO_{total}$ weight ratio (%). Here, in the SMS experiment, looking at soil solution, there was a fluctuation of total Cu (and active Cu) with exposure time: increase from 0 to 28 days followed by a decrease or stabilization, although low and not significant. Hence, the contribution of soluble Cu for toxicity differences seems relatively low in soil but the measurements at individual time points also provide a limited interpretation. Similarly, in vitro testing showed that the toxicity of the surface-modified CuO NPs was not directly linked to NP dissolution and subsequent Cu burden in cells (Líbalová et al., 2018).

The LUFA 2.2 soil CEC will allow the negatively charged -CIT and -ASC CuO NMs to attach to the existing cations (Na^+, Mg^+) while the polymeric -PEI and –PVP, due to their steric interactions and volume, may occupy most of the attachment sites with fewer number of molecules, potentiating the agglomeration process and release into the soil-solution of the remaining CuO NMs, making it less available for soil dwelling invertebrates in the immediate and with slower release during longer time.

Also, the lack of charge in the PVP-surface modified NMs may decrease its binding with soil particles, resulting in a gradual sedimentation overtime. This can still contribute to the toxicity in collembolans, as these feed on fungi in soil and can accumulate soil in gut (Fountain and Hopkin, 2005; Peijnenburg and Jager, 2003).

While it has been reported that the toxicity of CuO NMs is not solely due to dissolved Cu^{2+} but also due to the nanoform (McShane et al., 2014; Wang et al., 2016), there was no direct correlation between abundance levels and any of the copper fractions in soil or soil solution was observed. The amount of CuO present as NMs in the soil was not possible to determine due to technical difficulties/impossibilities, that is, the particle size was below the detection limit of 15 nm (Navratilova et al., 2015).

There are several factors in soil that combined determine the toxicity level of NM: from the materials life cycle side, longer exposure time has shown that toxicity differences become more pronounced at later periods; from the biological-ecosystem side, multispecies interactions contribute significantly for the final toxicity outcome and are not always predictable based on individual species testing, especially if few species are covered.

In terms of species weight in the SMS, *F. candida* was the one most driving the overall outcome, followed by M. *macrochaeta* (together with *H. assimilis*), basically for opposite reasons, the first had the highest abundance, the latter the lowest (2000 vs 20). Previous SMS results with exposure to Cu salt (Scott-Fordsmand et al., 2008; Menezes-Oliveira et al., 2013, 2014) and CuO NMs (Mendes et al., 2018), showed *E. crypticus* to be the species with highest weight (and also most sensitive) in the system. This seems related to the test-design where a range of higher concentrations were used, including higher ECx for *E. crypticus* and consequent high decrease in population numbers. The present surface modified CuO NMs test also shows *E. crypticus* was the most sensitive at 200mg/kg soil DW, this compared to the other species. The higher abundance (reproduction rate) of *Folsomia candida* has been reported before, as well as the sharp decline in abundance for *H. assimilis* and *M. macrochaeta* (Mendes et al., 2018). Testing effect of predation showed that all collembolan test species were a "likable" prey for *H. aculeifer*, but the smaller ones (*H. assimilis* and *M. macrochaeta*) were eaten in higher numbers (Mendes et al., 2018), this together with the fact that *M. macrochaeta* has also lower mobility (Ponge et al., 2006) makes them an easier target. *F. candida* high reproductive rate compared to *H. assimilis* and *P. minuta* (see Figure 3), allows it to be the most abundant within the ecosystem, even in the presence of a predator such as *H. aculeifer* (Figure S2). This has been observed with Cu in other SMS studies (Mendes et al., 2018; Scott-Fordsmand et al., 2008; Menezes-Oliveira et al., 2013, 2014; Cortet et al., 2003).

The predator species, *H. aculeifer*, shows increase in abundance from day 28-56, at the same time that *P. minuta*, *H. assimilis* and *M. macrochaeta* decrease, hence it must be feeding on *E. crypticus* and *F. candida*. Furthermore, the higher performance of *E. crypticus* compared to the collembolans (except *F. candida*) may promote higher sensitivity of other prey species: Cortet et al. (2003) showed that the presence of *E.*

crypticus greatly reduces the available soil constituents compared to a collembolan only SMS.

The species' relative distribution in the soil layers (Figure S3) shows an overall tendency for higher numbers in the top and middle layer. This could suggest a relation with soil compaction, higher at the bottom. No predator-prey cues relationship has been observed in previous studies (Baatrup et al., 2006; Pfeffer and Filser, 2010), with their encounter being a result of random search.

Individual species testing showed also –CIT and –ASC to be the most toxic, although results differed from the SMS [(EC50 (IS) > EC50 (SMS)], clearly showing the impact of species interactions. To note that from the IS range finding tests that adult survival was affected mostly for *P. minuta* and *H. assimilis* (and not for *F. candida*) – these are important species-specific mechanisms of response which contribute to the overall observed ecosystem impact.

Measurements of internal body Cu concentration showed that depending on the surface modified CuO NMs time to reach equilibrium or steady state was faster (-ASC) or slower and continuously increasing until day 84 (-PVP). The maximum internal concentration was ca. 250µg Cu/g body DW in the SMS, slightly higher for higher (300µg Cu/g body DW) concentrations in the IS exposure. This suggests that the coating interfere with the release of Cu^{2+} and/or the activation of copper regulators and detoxification mechanisms in the organisms (Ardestani et al., 2014; Ardestani and van Gestel, 2014).

In terms of interactions in soil and extracellular matrix, -CIT and –ASC are more expected to adhere to biological molecules and form e.g. protein coronas which will promote cell internalization (Verma and Stellacci, 2010). Another mechanism can be disrupting the membrane and cell stability due to the negative charge, leading to a more immediate toxicity potential, compared to -PVP (Cvjetko et al., 2017). The differences in internal Cu concentration between -CIT and -ASC CuO NMs exposure may be due to the distinctive roles of citrate and ascorbate in the organism. While both may be actively transported into the cell, ascorbate can actually be passively transported into the cytoplasm, being presented as both a receptor agent for the NM (Horemans et al., 2000; Lane and Lawen, 2009), allowing its internalization into the cell, and an antioxidant defence agent to the increasing intracellular Cu concentration (Drążkiewicz et al., 2003;

Finkel and Holbrook, 2000; Thounaojam et al., 2012; Patananan et al., 2015), having a detoxifying action (Wang et al., 2013). On the other hand, the increasing citrate availability will not only decrease the cellular pH, but will also interfere with the mitochondrial metabolic process (Gnoni et al., 2009; Mycielska et al., 2009), leading to an increased ROS production (Soenen et al., 2010; Sun et al., 2010), coupled with the copper-induced Fenton reactions that also result in an increased ROS concentration (Chibber and Shanker, 2017) and should have a greater cytotoxic effect compared to the ascorbate.

Polymeric coatings have also been shown to promote CuO NMs uptake in other species such as *C. reinhardtii* (Perreault et al., 2012), and also here –PVP and –PEI showed relatively higher uptake than PRI.

While on the individual species testing, for similar exposure concentrations to the SMS, -CIT resulted in a lower internal copper concentration than -ASC in *F. candida*, PEI- resulted in the higher internal Cu. This difference suggests that the presence of other species and the organism life stage interfere with Cu intake.

Overall, toxicity increased with exposure time, similar to what has been previously reported (Mendes et al., 2018). This is further supported by long term studies with *E. crypticus* exposed to CuO NMs in multigenerational or full life span tests (Bicho et al., 2017; Gonçalves et al., 2017). Hence, the current framework including standard short-term exposure should move towards longer term testing.

5. Conclusions

Ecotoxicity differed between the various surface modified CuO NMs: –CIT and –ASC were relatively more toxic than the other (-PEI << -PVP ≤ PRI), which correlated with zeta potential: -PEI (+28mV) caused the least impact, -ASC and -CIT (-17, -18mV) the most, while PVP and PRI (-8, -9mV) caused an intermediate response. Although a complex matrix like soil adds many possible interactions there was a pattern between zeta potential and biological effect. The contribution of soluble Cu for toxicity differences was too low to explain the observed differences. Internal Cu body burdens showed that time to reach steady state was faster (-ASC) or slower (-PVP) and continued to increase until day 84 depending on the coating. This suggests that the coating interfered with the release of Cu^{2+} and/or the activation of copper regulators and

detoxification mechanisms in the organisms. Toxicity differences become more pronounced at longer exposure time later periods and multispecies interactions contributed significantly for the final toxicity outcome. The current risk assessment framework should move towards requiring longer term testing and a multispecies approach (or at least increase the number of individual species testing coverage required).

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Supporting Information

Figures (S1-S5) and tables (S1-S2) are provided as supporting information.

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Supplementary Information

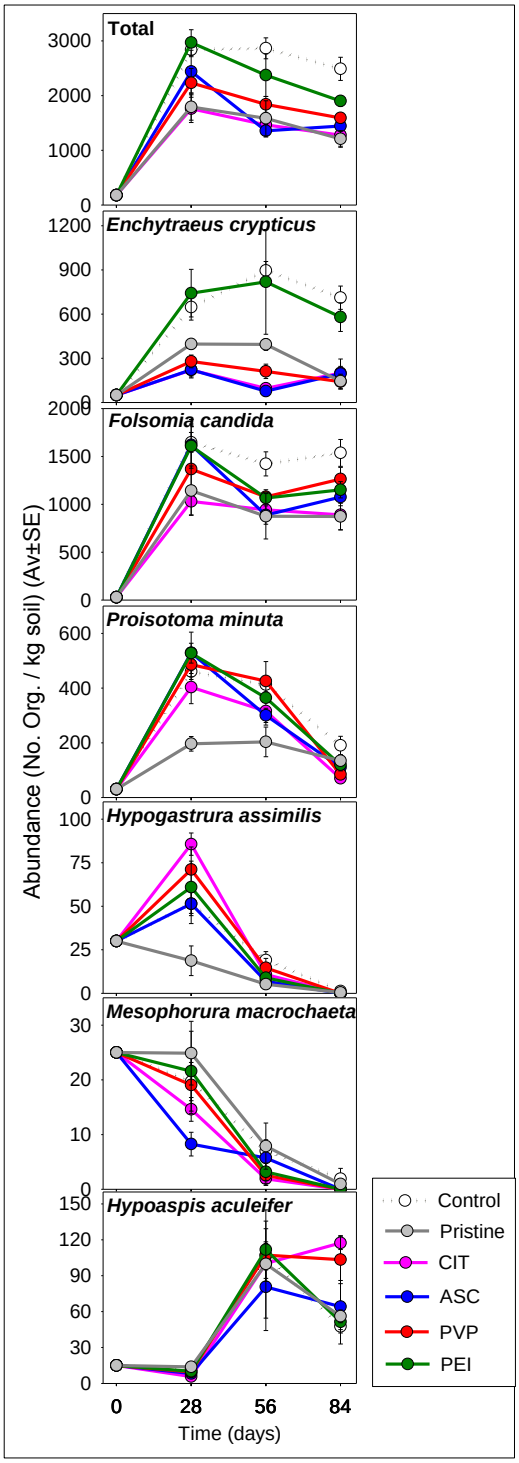

Figure S1: Total and individual species abundance obtained from a soil multispecies test system experiment performed in LUFA 2.2 soil spiked with CuO NMs surface modified (0-200mg CuO NMs/kg soil DW) and three exposure periods (28, 56 and 84 days). Results are expressed as average ± standard error (AV±SE, n=4). CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine.

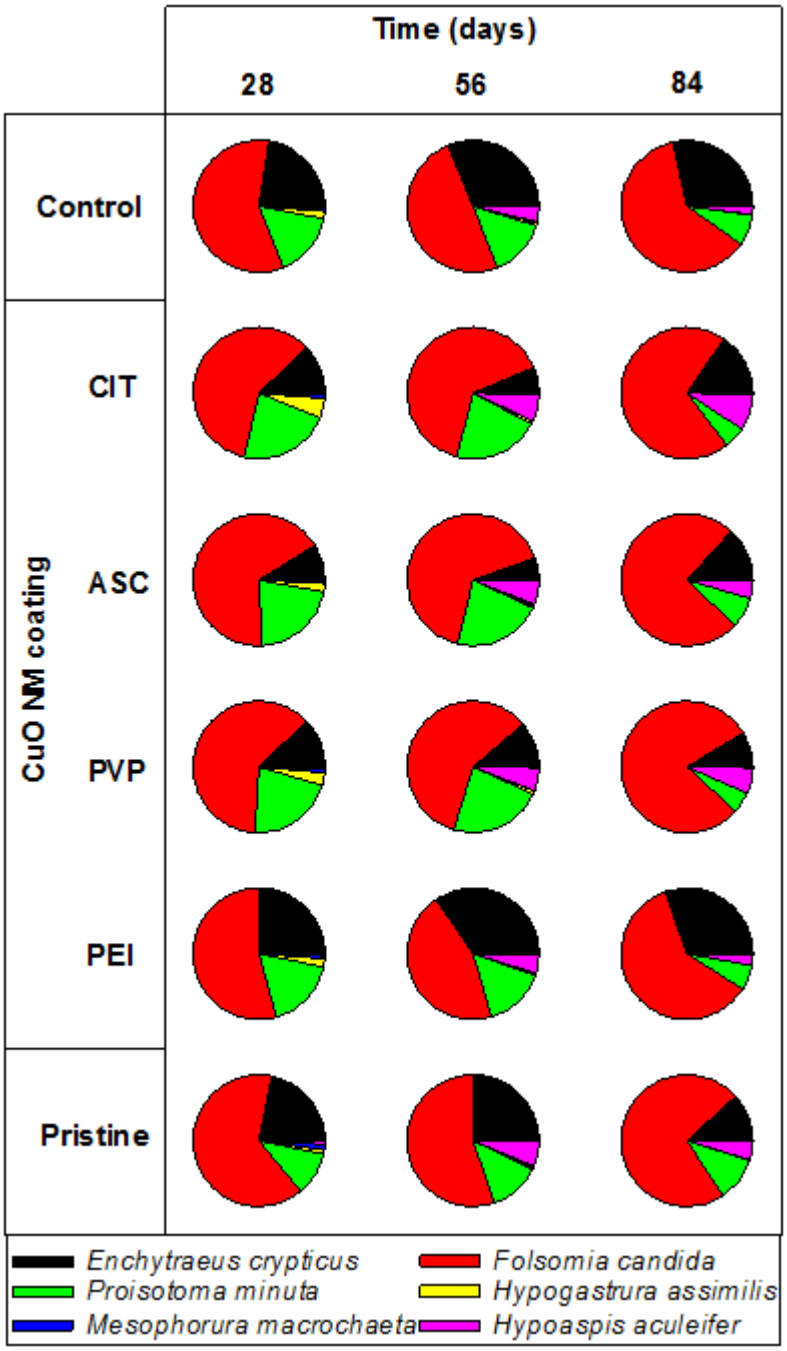

Figure S2: Pie chart with the composition (based on number of organisms) of the soil multispecies test system (SMS) obtained from a soil multispecies test system experiment performed in LUFA 2.2 soil spiked with different-surface modified CuO NMs (0-200 mg CuO NMs/ kg soil DW) and three exposure periods (28, 56 and 84 days). Results presented as average % of total population (n=4). CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine.

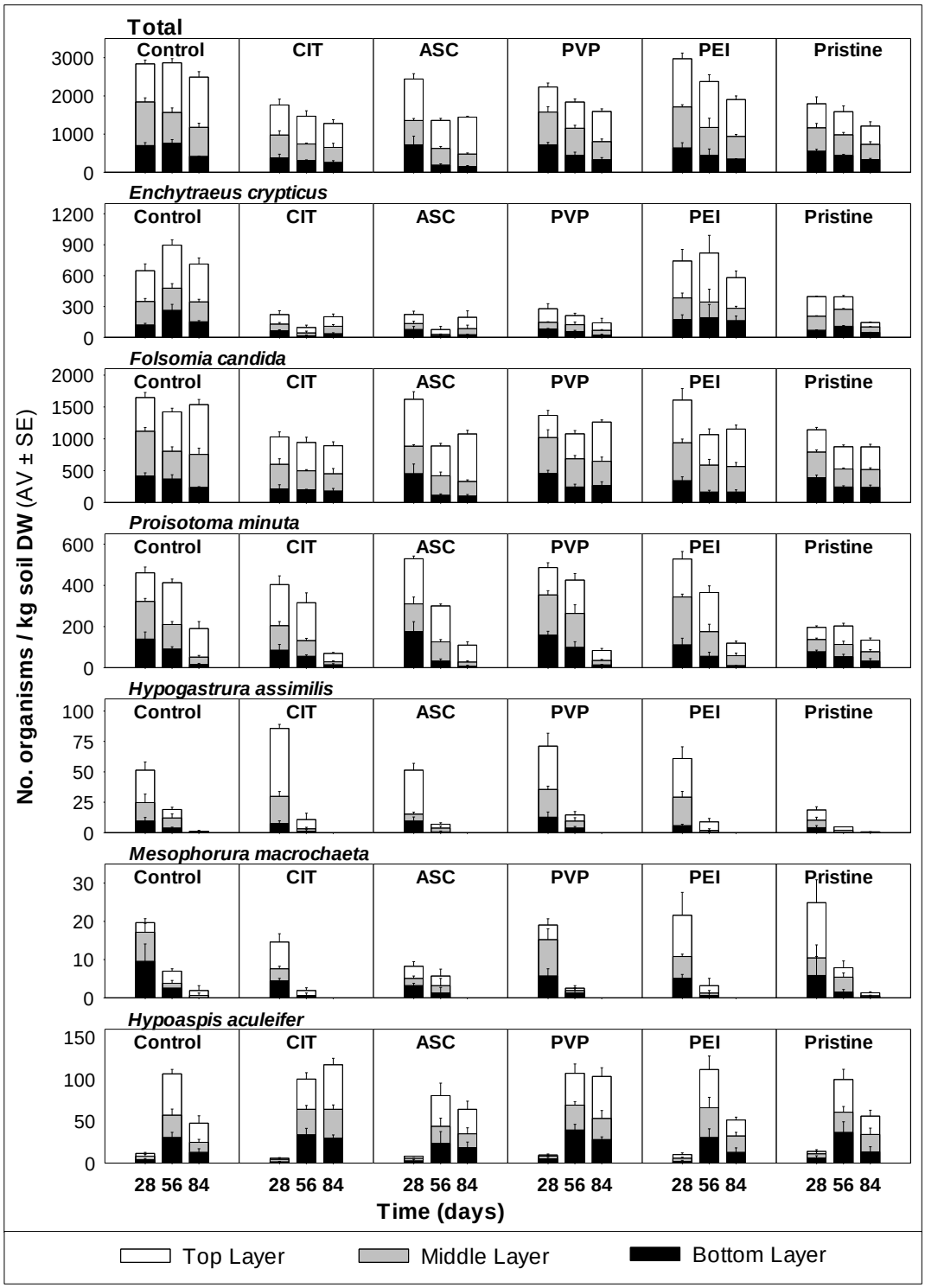

Figure S3: Species abundance layer distribution of the soil multispecies test system (SMS) obtained from a soil multispecies test system experiment performed in LUFA soil 2.2 spiked with different-surface modified CuO NMs (0-200 mg CuO NMs/ kg soil DW) (+ Control) and three exposure periods (28, 56 and 84 days). Results presented as number of organisms per layer in a kg soil DW (average ± standard error, n=4). CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine.

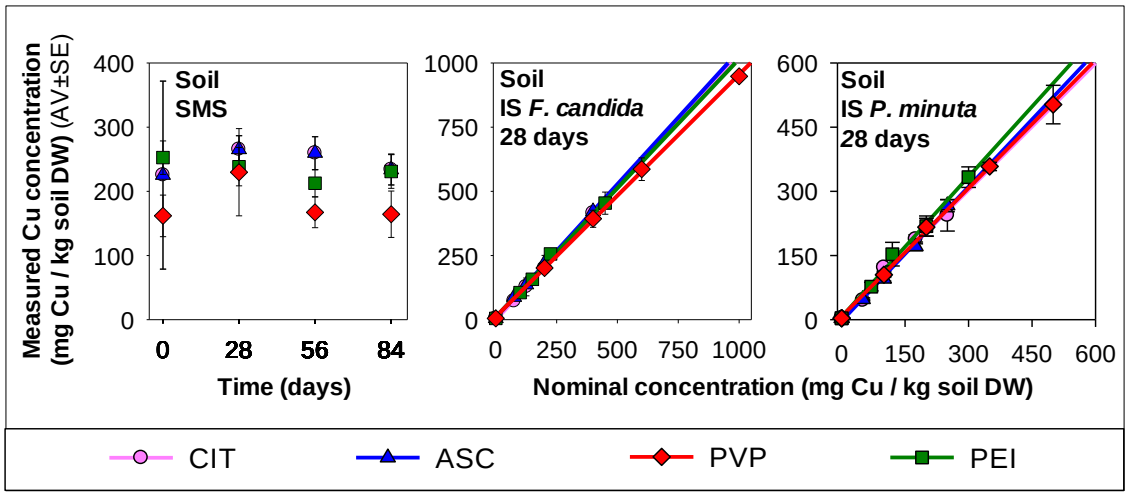

Figure S4: Measured total copper concentrations in soil from the soil multispecies test system (SMS) experiment performed in LUFA 2.2 soil spiked with different surface modified CuO NMs (0-200 mg CuO NMs/ kg soil DW) and three exposure periods (28, 56 and 84 days) and from the individual standard tests with *F. candida* and *P. minuta* exposed to different surface modified CuO NMs. Results presented as mg Cu / kg dry soil (average ± standard error, n=4). CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine.

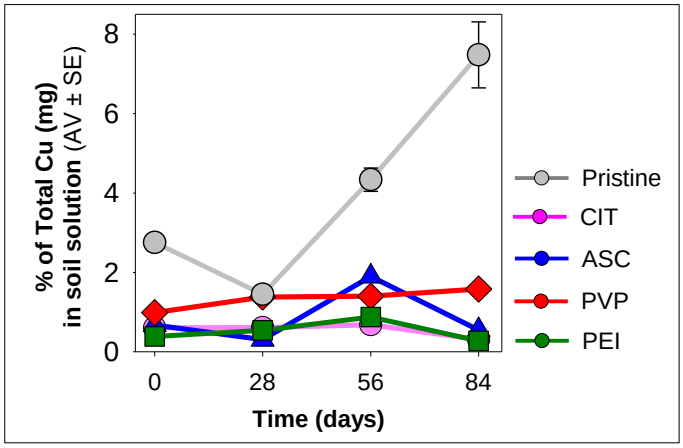

Figure S5: Relative concentration of active total Cu in soil solution of the soil obtained from a soil multispecies (SMS) test system experiment performed in LUFA 2.2 soil spiked with different surface modified CuO NMs (0-200 mg CuO NMs/ kg soil DW) and three exposure periods (28, 56 and 84 days). Results presented as % Total mg Cu measured (average ± standard error, n=2). CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine.

Table S1. Summary of the Main Properties of the Tested CuO NMs powder as synthesised.

Properties	**CuO NMs**
Manufacturer	Plasma Chem
CAS number	1317-38-0
Primary size distribution (average)	3-35 (12)
Mode (1st quartile - 3rd quartile)[nm]	10 (9.2-14)
Shape	Semi-spherical
Average crystallite size [nm]	9.3
Crystallite phases (%)	Tenorite 100%
Dispersability in water: D50 [nm];	139.5 ± 4.6;
average agglomeration number (AAN)	346
Dispersability in modified MEM: D50 [nm];	85.2 ± 2.7;
average agglomeration number (AAN)	77
Z-potential in UP water [mV]	+ 28.1 ± 0.6
Isoelectric point [pH]	10.3
Photocatalysis: photon efficiency [unitless]	1.5 x 10-4
Specific Surface Area [m2 g/1]	47.0 ± 1.7
Pore sizes [nm]	13.5 ± 1.6 (BJH)
	23.0 ± 0.9 (AVG)
Surface chemistry [atomic fraction]	Cu = 0.46 ± 0.05; O = 0.47 ± 0.05
	C = 0.07 ± 0.01
Chemical impurities [mg kg/1]	Na: 505 ± 30; Pb: 36 ± 2 Ag: 13 ± 4

Table S2: Significance (p value) as calculated by the Monte Carlo permutation tests, done by testing each CuO NMs coating against the control for each time point of the soil multispecies test system (SMS) experiment performed in LUFA 2.2 soil spiked with CuO NMs surface modified (0-200mg CuO NMs/kg soil DW) during three exposure periods (28, 56 and 84 days). CIT: Citrate; ASC: Ascorbate; PVP: Polyvinylpyrrolidone; PEI: Polyethylenimine; PRI: Pristine (data normalised to control).

	CuO NMs				
Time (days)	CIT	ASC	PEI	PVP	PRI
28	0.002	0.002	0.952	0.13	0.002
56	0.002	0.002	0.158	0.002	0.076
84	0.002	0.142	0.204	0.002	0.002

Chapter six

General Discussion and Conclusions

General Discussion

Folsomia candida has been shown to be sensitive to any NM to which it has been exposed in this work, from silver to copper oxide-based NMs.

As pointed out in the introduction, the work revolved around four main pillars that support the development of toxicity of NMs to soil invertebrates: Time; Transformation on Exposure Media; Modification of NM Characteristics; and Species Interaction.

Time

NM effects are intrinsically connected with time. Throughout this thesis, in both Ag and Cu exposures, for similar concentrations, the salt form effects systematically took place before the NM effects, regardless of the timeframe of the study or the parameter measured.

This shows a time-dependency for the development of NM toxicity, related to the availability of the dissolved ionic form as the cause for longer-term toxicity. Similar and simultaneous responses were obtained when the NM concentration was 3-4 fold higher than the non-nano form. Considering the ionic form as the source of toxicity, it would indicate that only approximately 30% of the metal has been released/dissolved from the NM. This shows a lower release rate of ions from NM than from non-nano (salt) counterpart, leading to an increase in toxicity on longer-term exposures (Gomes et al., 2015). Furthermore, reports of no effects due to NM exposure are set within standardized testing guidelines, limited to 28 days (Noordhoek et al., 2018), while our studies have shown that responses at all levels take longer time for NMs than salt in *F. candida*. From this, we suggest that the required time period for NM testing with *F. candida* should be increased.

The use of a multigenerational exposure to evaluate the effects of $AgNO_3$ over the course of 6 generations showed an impairment in reproduction in continuous exposure but also the potential for transgenerational effects. After a reproduction rate decrease due to initial exposure to Ag, even in clean soil the reproductive output did not reach control levels. In the same study, the size of organisms decreased with the number of generations, suggesting adaptations in response to exposure to the Ag, in order to survive.

Transgenerational effects due to metals in springtails (Amorim et al., 2017) and in response to NMs in soft-bodied invertebrates (Bicho et al., 2017a; Schultz et al., 2016; Yang et al., 2016) have been suggested. The increased expression of metallothionein in response to the stressor (Amorim et al., 2017) is indicative of activation of the antioxidant defences, with the decrease in reproduction and/or as a possible trade-off, as reported in chapter three. Based on the later activation of metallothionein due to AgNM compared to $AgNO_3$ [chapter two], it could be hypothesized that the antioxidant defences would be activated later and the changes on the organism and population level would be reflected only on later generations. As the metal internal concentration is increased due to CuO NM exposure [chapter four], it is suggested that the exposure to AgNM would permanently activate the antioxidant defences, with a greater hazardous potential with an increasing number of generations (Shaw et al., 2017). Studies with iron NMs and *C. elegans* have shown that the parental generation exposed to NMs resulted in an increased iron internal content in the following non-exposed generations (Yang et al., 2016). It is therefore possible that the same occurs in springtails.

Due to the NM impact on longer-term exposures and that can only be effective on later generations, the development of epigenetic alterations (Klosin and Lehner, 2016) and its assessment (Noordhoek et al., 2017) can be used to evaluate or predict the impact of NMs (Vandegehuchte and Janssen, 2014).

In this work, the largest timeframe tested was 186 days, with *F. candida* adapting to survive, whilst after 84 days exposure to CuO NMs in a multispecies design, *H. assimilis* and *M. macrochaeta* became extinct, while studies with 140 days and one-year old NM-contaminated soil have reported effects on *F. candida* (McKee et al., 2017) and *E. fetida* reproduction respectively (Diez-Ortiz et al., 2015). It should be important to assess in a multispecies system design up to a year the response of the soil invertebrate ecosystems, whether all the species would go extinct or if any of the species would adapt to the continuous exposure and NM transformation.

The use of organism level endpoints such as size contributes to a better understanding of population dynamics in response to a stressor, not limiting to an increase or decrease in the number of organisms, as current standard tests follow.

Transformation on Exposure Media

The availability of the NMs to organisms is a determining factor in toxicity. This depends on the fate of the NMs in the exposure media. By AAS techniques, Cu was quantified in soil samples and it was observed that nearly all was found in the soil particle matrix, while a very low percentage was measured in the soil solution. This was similar to the levels observed in soil spiked with the salt form of Cu. With the addition of coatings to the CuO NM, for similar concentrations, the percentage of total Cu in soil solution was higher than from the pristine form, nonetheless remaining quite low (<3%). Relatively low rates of total metal in solution vs soil matrix were also observed for ZnO NMs and Zn salt (García-Gómez et al., 2014), while the fate of AgNMs depended on the soil characteristics (Hedberg et al., 2015).

Regarding the quantification of the active ionic Cu^{2+}, our results indicate that the % of free ion available in soil solution, compared with the total in sample was very low, albeit higher at earlier timepoints if the NM was coated (max 0.025%). However, it was not possible to draw a direct correlation between the dissolved Cu fraction and the effect on the multispecies system. Other authors have used the same technique to quantify dissolved Cu in soils, the soil used did not have the same characteristics (McShane et al., 2014). One factor to take into account is the percentage of organic matter (1.77% in LUFA 2.2 soil) that may bind with the existing cations or aggregate with the NM itself, limiting the available Cu ionic form (Djae et al., 2017). Besides the organic matter in the soil, the addition of other ions released from the coatings (Na^{+}; O^{2-}) affects the actual availability of Cu^{2+} in the soil solution and the measurements. Additionally, Shane et al. (2014) also refer that Cu^{+} activity is in considerably log units lower than Cu^{2+}, possibly resulting in an underestimation of the dissolved Cu in the soil-solution.

No increase in total and active Cu concentration in soil solution was observed after 84 days, however changes in the internal copper content and effects on abundance were observed. These findings allow the supposition that the main source of toxicity from these NMs is not related with the free active ionic form dissolved into the soil solution but due to interactions of the NM with the soil matrix. Copper speciation as a result of sorption with organic matter in the soil matrix, after the release of Cu from the NM, is likely to occur (Julich and Gäth, 2014). Which complexes are formed, and their stability need to be studied to determine the actual availability to organisms.

Modification of NM Characteristics

As mentioned, modification (coating) of CuO NMs alters its fate in the exposure media, as the composition of the NM surface will determine the interactions and dynamics, not only due to the organic matter, in soil and soil solution (Baalousha et al., 2018).

From the results in chapter five, there is a correlation between the acquired zeta-potential of coated CuO NMs and their toxicity in a multispecies environment. It has been reported that both silver and copper oxide NMs have a higher dissolution rate in most of the aqueous media tested when coated with citrate (Gondikas et al., 2012; Ortelli et al., 2017). Dissolution of CuO NMs has been reported to increase in aqueous media with increased concentrations of citrate (not present as a coating), forming copper citrate (Peng et al., 2017). This may facilitate the metal uptake as ionic form and consequently its more immediate toxicity

Comparing the coatings used, the CuO NMs coated with polymeric structures (PVP and PEI) are less toxic than coated with CIT and ASC. In this regard, it is possible that the addition of coatings made up of monomers that have an important biological role in organism, e.g. citrate, as an essential part of the Krebs cycle (Iacobazzi and Infantino, 2014), or ascorbate, with a predominant role in antioxidant defences (Padh, 1990), may facilitate the recognition and uptake of CuO NMs.

Based on the work, it is suggested to use the zeta-potential of the CuO NMs as a parameter to predict its impact on long-term exposures. However, the mechanisms by which the toxicity is developed are not yet clear, as similarly charged NMs have different accumulation patterns in *F. candida*. It is possible that the biological role of the coatings constituents can determine the specific mechanisms that lead to toxic effects.

As *F. candida* is shown to uptake metals by way of the cuticle or the ventral tube through soil ingestion (Fountain and Hopkin, 2005), the uptake mechanism that results in an increased internal metal concentration after CuO NM exposure is not clear yet. One possible mechanism for metal release from NMs inside the organism is by degradation in lysosomes (pH = 4), as a lower pH is known to accelerate the dissolution process, compared to the cytoplasm (pH = 7.4) and the soil (pH~6) values (Peng et al., 2017). Whether the uptake is done in the form of NM and later transformation inside the

organism, from a copper-soil particle complex or free Cu itself has been a source of discussion and remains to be seen (Ardestani and Gestel, 2012; McShane et al., 2014; Gomes et al., 2015)

These possible uptake mechanisms for CuO NMs (coated and non-coated) by *Folsomia candida* and consequences on a cellular are represented in Figure 1.

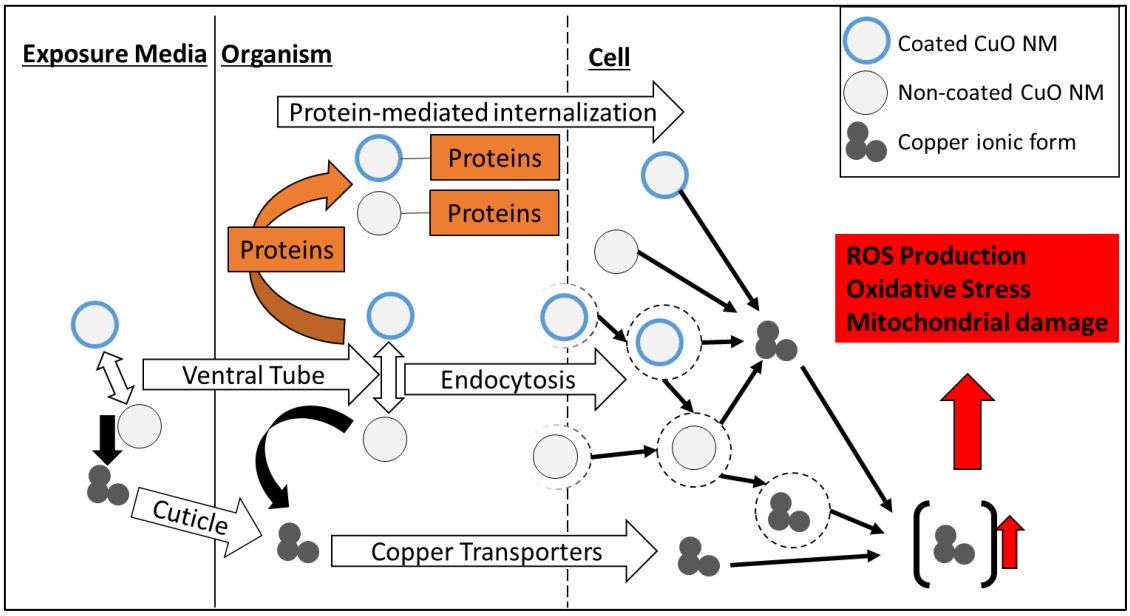

Figure 1: Schematic representation of the possible pathways for Cu internalization and increasing availability to the organism after exposure to coated CuO NMs.

While it was possible to observe an increase in internal Cu concentration after exposure, the absence of information regarding the quantification of dissolved metal and its nano form in both the soil and the organism was a limitation due to particle size (Navratilova et al., 2015). This should be addressed in any future work. However, in the literature, no information on quantification of nanomaterials with size below 20 nm in soil was found.

Also in this work, it has been shown that the nano and dissolved forms of Ag result in different antioxidant defence activation patterns, albeit in short exposures (up to 10 days). It is possible that the presence of coating molecules can also affect the activation of antioxidant defences. This should be tested on more complex exposures, such as the multispecies system, in order to better understand the path to NM toxicity.

Species Interactions

Species interactions have been shown in this work to have an essential role in the impact of CuO NMs. In this sense, multispecies system tests are a decisive tool as its design can cover several timepoints and therefore observe several species population over the course of more than one generation and compare several conditions in the same experiment (concentration range; nano vs non-nano; different coatings), according to the aim of the study.

With the use of multispecies system testing, effects of CuO NMs were observed in different species at different degrees. While soft-bodied organisms were highly affected, their ranking amongst species was not the same for all exposures scenarios. Predatory mites were shown to be the least affected by the exposure, based on the EC50. This is due to their function as predator in the ecosystems. As their main energy source is not contained in the soil matrix, e.g. microbiota, they are not directly affected by the soil spiking with the contaminant. The size and quality of the energy source (preys) is decisive for the fitness of *H. aculeifer*, as shown by Heckmann (Heckmann et al., 2007).

The most affected collembolan species in any of the multispecies and single species experiments were the ones with smallest organism size. This is understandable as the mg Cu available / mg body tissue is higher for smaller organisms than for larger such as *F. candida* or *P. minuta*. Additionally, the results of a small-scale predatory test, showed that predatory mites tend to feed more on smaller sized collembolans, with this tendency being increased after exposure to both salt and NMs. Therefore, in an ecosystem, the stressor effect is a synergistic effect of the exposure and the presence of a predator.

What is the weight of each of these factors in the decrease in species' abundance is up for debate. However, the decrease in abundance of *H. aculeifer* when *E. crypticus*' abundance is not decreased, even when exposed to coated NMs, suggests that the exposure is the first factor in species survival, with the predatory frequency increasing due to a weakening (mobility, size) of the prey species.

As the prey source has been shown to have an impact on mite reproduction, the use of multispecies testing, aided by the predation/feeding test can provide information on the feeding preference of *H. aculeifer*. As such, the tendency to feed on smaller species, the

increase in abundance with the decrease in smaller collembolans species and the decrease in mite abundance when *E. crypticus* did not decrease were considered. Therefore, a ranking of species "preference" for mite feeding is proposed, based on the species used (Table 1).

Table 1: Species ranking for *H. aculeifer* feeding preference based on multispecies testing and predation on collembolan testing.

Ranking	**Species**	**Multispecies testing**	**Predation on collembola testing**	**Other information**
1.	*E. crypticus*	*E. crypticus* abundance ↑ *H. aculeifer* abundance ↓ (PEI-coated NM exposure)	-	Soft-bodied organism
2.	*M. macrochaeta*	Abundance ↓with time Extinction	Highest predated organism	Smallest species
3.	*H. assimilis*	Abundance ↓with time Extinction	Second highest predated organism	
4.	*P. minuta*	*E. crypticus* abundance ↑ *H. aculeifer* abundance ↓ *P. minuta* abundance ↓ (PEI-coated NM exposure)		
5.	*F. candida*		Lowest predated organism	Largest collembola species

Although the discussion has focused on the bioavailability and uptake of CuO NM by the soil invertebrates, there are other indirect pathways for toxicity. One is the possible impact of the CuO NM in the soil microbiota, as it is the main source of energy for collembolans and enchytraeids, supporting the soil ecosystem (Cortet et al., 2003, 2006). It has been shown to be affected by metals, both as nano (Nogueira et al., 2012; Rousk et al., 2012; Concha-Guerrero et al., 2014; Moore et al., 2016) and non-nano (Henriques et al., 2015). As such, added to predation and direct exposure, the lack of food source can lead to a decrease in abundance, due to decreased reproductive output.

The extinction of two species in the multispecies testing shows that, while NMs may have a hazardous effect on individual species, when together with species interaction

and longer-term exposures, they can lead to species extinction and consequently change the ecosystem composition and its dynamics. In Figure 3 it is illustrated the time-dependent potential impact of NMs in the microbiota and resulting lack of energy source to the soil invertebrates, altering the interaction between species, promoting predation and competition. In turn this will reduce the species diversity, leading to changes in the ecosystem composition and dynamics (Figure 3).

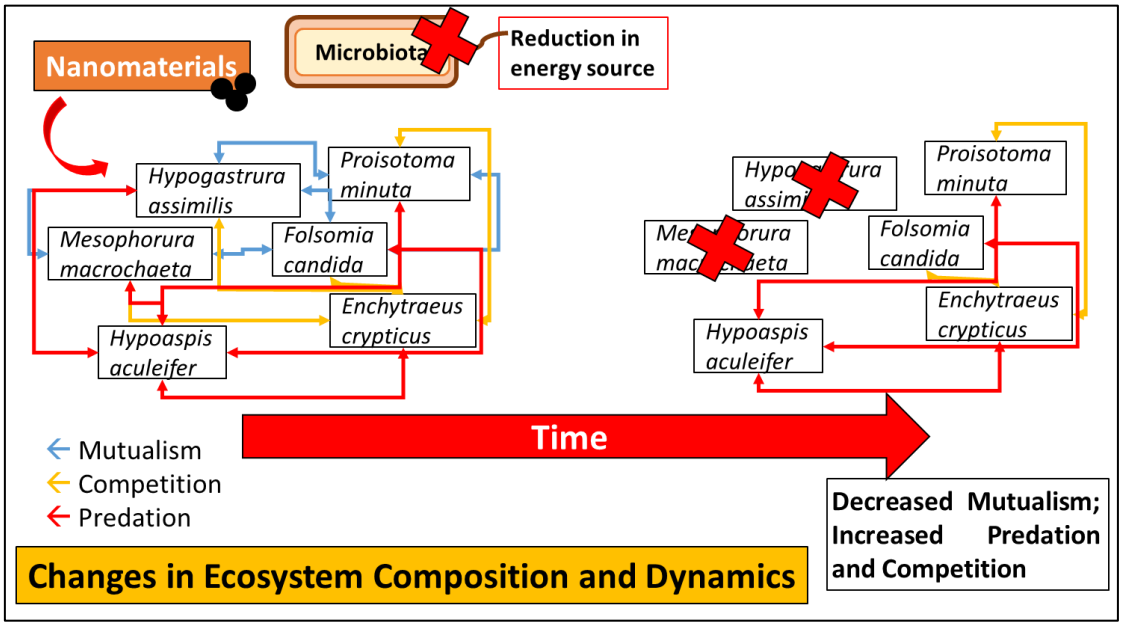

Figure 3: Schematic representation of the synergistic effect of NMs and species interactions mutualism, predation, competition) in a soil invertebrate ecosystem overtime. Lines represent species interactions, with the prevalent interactions represented by thicker lines. Blue – mutualism; yellow – competition; red - predation

Added to the observed effect on several species, by the use of standardized testing, it was shown that the effects can be life stage dependent, as based on populational level endpoints, with juveniles being more sensible than adults, regardless of the pristine or coated CuO NM. This has been reported in *E. crypticus* exposed to non-coated CuO NMs (Bicho et al., 2017b). Due to the clear difference in size between life stages, it is suggested that the higher mg available stressor / mg body tissue in earlier life stages may be the main contributing factor for this difference.

From this discussion the main idea is that a multi-endpoint evaluation with the combined use of an increased time-length standardized and multispecies testing should be applied to fully understand and predict the effect of NMs in the soil ecosystem. In Figure 4 the various endpoints measured in this work, their distribution on the different

organizational levels, the timeframe required for assessment according to the type of exposure (nano or non-nano) and the connections between different level endpoints are illustrated (Figure 4).

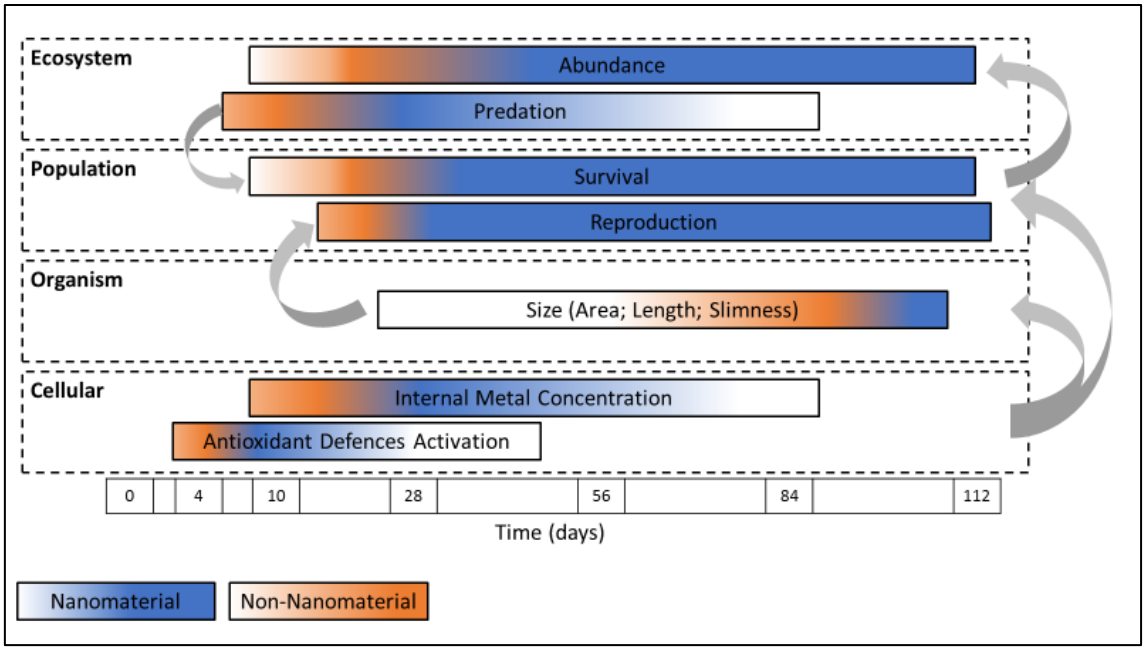

Figure 4: Schematic representation of the parameters evaluated at different organizational levels and the minimum required time length for assessment of NM and non-NM effects on *Folsomia candida*. Changes on a basic (cellular) level have a consequence on consecutively more complex levels.

Exposure to $AgNO_3$ over several generations lead to the development of transgenerational effects in *Folsomia candida*. Firstly, this shows the ability of the springtails to adapt to survive. Secondly, the use of this experimental design with NMs would contribute to environmental risk assessment and should focus on the evalution of epigenetic changes.

Current standardized testing guidelines for *Folsomia candida* exposure can be updated in order to consider the exposure to NM. From this work, we can propose the addition of size evaluation as an important parameter for toxicity testing. Also, we propose the increase from 28 days from the current testing timeframe. This should overlap the current limitation that most NM effects are not shown in such short period.

Lastly, the use of multispecies system testing has been shown to be essential to evaluate the contribution of species interactions and soil-NM transformation dynamics to CuO NM toxicity.

In the current thesis, this approach allowed the correlation between the CuO NM zeta-potential and toxicity to soil invertebrates. This information can be used in future studies to predict the level of toxicity of nanomaterials.

The combined use of these with current standardized testing, together with soil microbiota descriptors, in a multi-endpoint approach similar to this thesis can properly evaluate and predict the impact of NMs to soil invertebrate ecosystems.

This will provide valuable information for the current and future development of safer-by-design NMs (Nowack, 2017; OECD, 2017a; Scott-Fordsmand et al., 2017) that can be used in industry and introduced as part of nanoapplications into the mass market (OECD, 2017b), as well as contributing to policy making processes regarding industial and environmental regulation (Foss Hansen, 2017; Karjalainen et al., 2017; Malsch et al., 2018; OECD, 2016; Rycroft et al., 2018).

Conclusions

From the work presented in this thesis, the main ideas are highlighted:

The selected NMs effects on soil invertebrates are time-dependent and effects on the cellular organizational level on short time exposure are cumulative and result in effects on the ecosystem organizational level on longer term exposures. While non-nano exposure results in more immediate effects, NMs have a longer-term hazardous potential.

The acquired zeta-potential of CuO NMs can be used to predict its impact, with negative-charged NMs having higher toxicity on long-term exposure than positive-charged NMs, with non-charged NMs developing a more imeediate and continuous effect. The toxicity of coated CuO NMs to soil organisms is likely due to transformation mechanisms that occur in the soil matrix or inside the organism, as both pristine and coated CuO NMs were virtually all present in the fraction of the soil particle rather than in soil solution.

Species interactions and NM exposure have a synergistic effect on soil invertebrate ecosystems, with predation having an important role in regulation of species abundance and possibly extinction, changing ecosystem composition and dynamics.

As such, the Soil Multispecies System (SMS) testing is an important tool for risk assessment in soil invertebrate ecosystem, as it provides further information that the current single species standardized testing, and can be used in further studies on the NM characteristics that contribute to NM toxicity

Study Recommendations

Based on the work and experience obtained in this thesis, the following suggestions are made for a proper NM risk assessment, in order to obtain the full information for the development of safe-by-design NMs. This information will be useful in policy-making processes regarding industrial, environmental and NM regulations:

Increase the exposure period, updating the current standardized protocols for soil invertebrate testing, accommodating it for NM exposure.

Application of the multi-endpoint approach presented in this thesis, using both SMS and standardized testing to other fully characterized NMs, for assessment of other characteristics (size/shape) that can contribute to NM toxicity.

Quantification of the actual nanoform in both exposure media and in organisms, along with the application of the oxidative stress biomarkers on more complex exposures (SMS), for a better understanding on the mechanisms that result in NM toxicity.

Further explore the mechanisms of NM toxicity on collembolans, both on short and long-term, by assessing the effects on a sub-cellular level: measuring the gene expression, epigenetic changes and potential DNA damage.

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